Growth, Survivability, and Reproductive Effects of Pulse-Dosed Endosulfan on *Jordanella floridae* (Florida flagfish) Over One Complete Life-cycle.

by

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Abstract

Endosulfan is a commonly used organochlorine in Durham Region, Ontario Canada which has known toxic effects on non-target organisms including fish. This research investigated the effects of endosulfan on Florida flagfish (*Jordanella floridae*), using both continuous and pulse-exposure. The 96 hour continuous exposure LC_{50} in larval flagfish was 4.35 µg/L; sub-lethal observations included hyperactivity, convulsions, and some axis malformation. The effects of a 4 hour endosulfan pulse-exposure on 7-8 day-old larval growth, reproduction, and survivability were investigated over one full life-cycle. The 4 hour pulse-exposure LC_{50} value for larval flagfish was 49.7 µg/L; there were no growth or reproductive effects of endosulfan pulse-exposure up to the highest exposure concentration of 10 µg/L. Thus, the life-cycle 4-h pulse-exposure no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were 3.2 and 10 µg/L endosulfan, respectively, due to significantly higher mortality.

Keywords: Endosulfan, Life-cycle, Reproduction, Flagfish

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List of Abbreviations

CC- Carrier Control

CW- Control Water

CCME- Canadian Council of Ministers of the Environment

d- day

dph- days post hatch

GABA- gama-aminobutryric acid

GSI- gonadosomatic index

h- hour

HSI- hepatosomatic index

LC_{50}- lethal concentration at which 50% of the test organisms would be killed

LOEC- lowest observed effect concentration

MS-222- tricane methane sulphonate

NOEC- no observed effect concentration

PCP- pentachlorophenol

ppb- micrograms per liter (µg/L)

ppm- milligrams per liter (mg/L)

TCB- 1,2,4-trichlorobenzene

TCE- trichloroethylene

1.0 Introduction

Organochlorine insecticides have been used around the world for many years for public health and agricultural production (Bradbury *et al*., 2008). These insecticides can be divided into four distinct structural classes; chlorinated ethane derivatives, cyclodienes, polychlorobornanes, and lindane (Bradbury *et al*., 2008). The use of these organochlorine insecticides has decreased significantly in recent years because of their toxic nature and ability to persist in the environment (Bradbury *et al*., 2008).

The cyclodiene insecticide endosulfan is still currently used as an alternative to some of the harsher organochlorine insecticides because it is less persistent in the environment. However, it is much more toxic to non-target organisms such as fish (Goebel *et al*., 1982; Harris *et al*., 2000). The toxic actions of endosulfan have been proven through documented fish kills. In Prince Edward Island there was a 90% mortality in threespine stickleback (*Gasterosteus aculeatus*) due to contamination from an aerial application of endosulfan (Ernst *et al*., 1991; Harris *et al*., 2000). In Ontario, an accidental spill in North Thames River causing concentrations of 0.096-0.26 mg/L caused 300-400 dace (*Chrosomus*), white sucker (*Catostomus commersonii*), rock bass (*Ambloplites rupestris*), and other species to die (Harris *et al*., 2000).

Endosulfan has been classified as an endocrine disruptor and has demonstrated antiestrogenic effects in fish with *in vivo* studies, however it has also shown weak estrogenic effects through different *in vitro* studies, thus the classification and mechanism of endosulfan as an endocrine disruptor is still debatable (Chakravorty *et al*., 1992; Palmer *et al*., 1998; Soto *et al*., 1995; Sumpter and Jobling, 1995). Endocrine disruptors can interrupt hormonal pathways that are necessary for reproductive behaviours leading

to reduced fertility and egg production (Arcand-Hoy and Benson, 1998). Since endosulfan can be classed as a potential endocrine disruptor it is of importance to better characterize its potential reproductive effects on fish.

One way to effectively study reproductive effects in fish is to use a life-cycle study. Life-cycle studies cover a wide range of endpoints and can help to elucidate the long-term effects due to contaminant exposure (Miracle and Ankley, 2005). Much of the risk assessment of chemicals focuses on endpoints such as; survival, growth, and reproduction which can directly assess the status of a population (Miracle and Ankley, 2005). Thus, the main aims of this research and their foci are outlined below.

1.1 Aims

The first aim of this research project was to determine the 96-h continuous exposure LC_{50} value for larval flagfish; this provides a standard measure of relative toxicity, and helps establish the overall sensitivity of flagfish to endosulfan. The second aim was to gather relevant information about pulse-exposure effects of endosulfan to larval flagfish; providing necessary information to allow selection of pulse-exposure time period and concentrations for the full life-cycle study. The third and final aim was to study the effects over a full life-cycle of a 4-hour pulse-exposure of endosulfan to larval flagfish. The null hypotheses for the full life-cycle study are: there are no effects of endosulfan pulse-exposure on survival, growth, or reproduction of flagfish.

2.0 Literature Review

2.1 Toxicity Testing

Toxicity testing has a long history dating back to the 1800s; however it was not until the 1900s that fish toxicity testing became more prevalent (Rand, 2008). The use of fish for acute toxicity testing led to the creation of standard aquatic toxicity testing protocols in both the American Society for Testing and Materials (ASTM) and the American Public Health Association (APHA) (Rand, 2008). This standardization was necessary and has made it easier for people all over the world to perform aquatic toxicology experiments with more consistency. Scientists can compare results and findings from different laboratories, knowing that with the use of standardized protocols, it is the scientific findings that are being compared instead of possibly differences due to procedural execution.

It is important to note the main reason why toxicity testing is conducted, it is most often completed in order to determine the relative toxicity of chemicals, and to assess any biological effects (Rand, 2008). Specifically of importance to the research conducted is the toxicity of chemicals to non-target organisms, such as fish.

There are many different types of toxicity tests that may be performed. Different techniques and methodologies can be used depending on the type of effect or question that is being asked. Some of the common methodologies include acute, sub-chronic, or chronic studies in which fish have been exposed continuously, intermittently, or pulsed (McKim, 1977; Parish, 1985; Rand, 2008; Sprague, 1969).

Of specific interest to the research that was conducted for this study are acute pulse-exposure studies and chronic pulse-exposure life-cycle studies. Both methodologies were employed in this study and thus both will be further discussed.

2.2 The Use of Pulse-exposure and Full life-cycle Studies

In many studies pulse-exposures have been used to examine the effects of environmentally realistic exposures of chemicals to non-target organisms (Ashauer *et al*., 2006). Since exposures in the environment typically vary over time and often occur in pulses (short duration), research has been conducted to assess the effects of pulse-exposures on growth, reproduction, and survivability of fish. This use of pulse-exposure has been validated in many other laboratory studies (Barry *et al*., 1995; Williams and Holdway, 2000; Holdway *et al*., 2008).

Another important type of testing in aquatic toxicology is the full life-cycle study. It is generally agreed amongst aquatic toxicologists that full life-cycle toxicity tests are the most accurate way to establish the long-term environmentally safe concentrations of chemicals (McKim, 1995). Life-cycle experiments generally involve studying the effects of an exposure on the growth, survival, and reproduction of the species being exposed (McKim, 1995). They are accurate because they cover a wide range of developmental stages throughout the organisms life and can give detailed information about delayed effects that might be seen (McKim, 1995). There have been many life-cycle tests conducted over the years using many different species of fish such as; fathead minnows (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), brook trout (*Salvelinus*

fontinalis), flagfish (*Jordanella floridae*), and sheepshead minnow (*Cyprinodon variegates*) (Eaton, 1970; McKim and Benoit, 1971; McKim, 1995; Smith, 1973).

One important biotic factor to take into consideration is the age of the fish during exposure because it can have a large impact on the sensitivity of the organism to the toxicant (McKim, 1995). An important reference to note for the basis of much of the work completed in this thesis was by Holdway and Dixon (1986).

2.3 Fish Physiology

2.3.1 Basic Uptake and Elimination Physiology

Fish are important test species for assessing the effects of environmental contaminants at the biological and biochemical level of response within aquatic ecosystems (Van der Oost *et al.*, 2003). There are two mains reasons for looking at fish for the effects of environmental contaminants. One reason is they are located in many aquatic environments including both marine and freshwater ecosystems such as oceans, rivers, and lakes. Secondly, they are an important part of the aquatic food-web, and a source of consumption for higher level organisms (Van der Oost *et al.*, 2003). Not only do other organisms feed on fish, but they are a main source of food for humans (Van der Oost *et al*., 2003).

Fish can be exposed to various toxicants through either waterborne, food-borne, or sediment exposures (Kleinow *et al*., 2008). The main methods of absorption include direct uptake via the gills or skin, through ingestion of suspended particles, and through consumption of contaminated food (Kleinow *et al*., 2008; Van der Oost *et al*., 2003).

The distribution of toxicants within the fish once absorbed has been generally described by Kleinow *et al*., (2008). A toxicant is first presented to the absorbing epithelium in water or gut contents, followed by transport into the blood from across the epithelium. The contaminant is then incorporated into the blood, and transported from the blood into the site of action (organs, tissues). Next, biotransformation of the toxicant is completed in order to allow for easier excretion, however, sometimes the biotransformation can lead to similar if not more toxic compounds than the original toxicant that was absorbed (Van der Oost *et al*., 2003). Typically the liver is the most common organ involved in the biotransformation of xenobiotics and one of the major ways in which the toxicant is biotransformed is through creating a more hydrophilic compound (Van der Oost *et al*., 2003). Once biotransformation has occurred the toxicant is ready for excretion. The main methods of excretion are through transport and diffusion across surfaces, and through biliary, urine, or fecal routes (Kleinow *et al*., 2008).

2.3.2 Basic Reproductive Physiology

Fish reproductive physiology involves a complex interaction between external stimuli, hypothalamic, pituitary, and gonadal hormones (Kime, 1999). Some of the external cues that are specific for each species are temperature and photoperiod (Kime, 1999). These cues trigger the release of gonadotrophin releasing hormone from the hypothalamus, which then causes the release of gonadotrophin from the pituitary. Gonadotrophin is the primary hormone involved with reproduction (Arcand-Hoy and Benson, 1998; Kime, 1999). The gonadotrophins then help to stimulate the growth of the gonads and production of steroid hormones (Kime, 1993; 1999).

For female fish, the major ovarian estrogen is estradiol and for males the major testicular androgen is 11-ketotestosterone (Kime, 1993; 1999). The growth of the gonads can take up to several months before viable gametes can be produced, after which the estradiol and 11-ketotestosterone secretion stops and a secretion of progestogen starts (Kime, 1993; 1999). The progestogen helps to induce the final maturation of the sperm and oocyte (Kime, 1993; 1999). This system works by a feedback mechanism in the gonads that can signal back their status to the hypothalamus and pituitary (Arcand-Hoy and Benson, 1998; Kime, 1993; 1999) (Appendix 1).

Exposure to toxicants can elucidate reproductive effects. Some of the reproductive effects that can often be seen are changes in reproductive output, spawning behaviour, fertility and fecundity, hatching success, and survivability (Arcand-Hoy and Benson, 1998).

There are approximately 20,000 species of fish in the aquatic ecosystem, however not all of them are suitable test organisms (Bucheli and Fent, 1995). The cyprinodontidae family of fish have been found to be an excellent test species to be used for laboratory studies (Foster *et al*., 1969).

2.4 *Jordanella floridae* (Florida Flagfish)

2.4.1 Classification, Habitat, and Characteristics

The species *Jordanella floridae*, commonly known as Florida flagfish, is a killifish that is native to the central and southern areas of Florida (Foster *et al*., 1969), which can also be found along the coastal Gulf of Mexico (Mertz and Barlow, 1966).

Flagfish are commonly found in shallow, weedy, freshwater areas, which can have wide variations in temperature, and they have also been observed in slightly brackish water (Foster *et al.*, 1969; St. Mary *et al*., 2004). The flagfish belongs to the cyprinodontiform order of fish which can be further classified into the cyprinodontidae family of fish (Bonnevier *et al*., 2003).

They are an oviparous fish, which are highly sexually dimorphic (Foster *et al*., 1969). The secondary sex characteristic which are present in males is an alternating of red and yellowish-green stripes, and the female secondary sexual characteristics are the presence of a black ocellus on the dorsal fin and their colouring is much more of a yellowish-olive colour (Foster *et al*., 1969). The typical size for a male flagfish to attain is approximately 50 mm, whereas the females usually only attain a length of approximately 45 mm (Foster *et al.*, 1969).

2.4.2 Success / Validity as Test Species

The cyprinodontidae family of fish have been found to be an excellent test species to be used for laboratory studies. In particular, the flagfish has been utilized because of well known and distinctive behavioural patterns, ease of breeding (continuous) and husbandry (Foster *et al.*, 1969). Flagfish can be sexed within 60-90 days (under optimal conditions) and they also have a short life-cycle of approximately 90-120 days, making them an excellent test species because of their short time to maturation (Foster *et al*., 1969; Holdway and Dixon, 1986). This ease of use as a test species, and easy adaptability to aquaria has been demonstrated in other laboratory studies (Hale *et al*., 2003; Klug *et al*., 2005; Mertz and Barlow, 1966; St Mary *et al*., 2001).

2.4.3 Behavioural Characterization & Breeding Patterns

Flagfish have extensive behavioural characteristics and are the only species known in the cyprinodontidae family that show parental care (Bonnevier *et al*., 2003; Foster *et al*., 1969; Mertz and Barlow, 1966). Their behaviour has been characterized as reproductive, feeding, and comfort (Foster *et al*., 1969; Hale *et al*., 2003; Mertz and Barlow, 1966).

Flagfish reproductive behaviour begins with a male who is displaying his fins maximally in order to guard the nest (spawning substrate) (Foster *et al*., 1969; Mertz and Barlow, 1966). Next, a female that is ready to spawn approaches the male; the female's colouration has blanched at this point (Mertz and Barlow, 1966). The female then swims parallel to the male and uses her caudal fin to do a series of turns. When her caudal fin is facing the male (90º) she backs up towards him, which causes the male to circle around her, while she maintains the 90º angle to his side (Mertz and Barlow, 1966). This behaviour, called T-circling, can vary in length of time but when the female is physiologically ready and they are oriented properly to each other they move onto the next behaviour which is known as the clasp (Foster *et al*., 1969; Mertz and Barlow, 1966). The clasp position is the spawning posture in which the male bends to hold its body against the female during spawning (Foster *et al*., 1969; Mertz and Barlow, 1966). At this point both the male and female vibrate their caudal peduncles quickly but with low amplitude (Mertz and Barlow, 1966). Female daily egg production is influenced by the amount of food in the gut; the more food present the more pressure it has on the ovary, which can help in the expulsion of ovulated eggs (Foster *et al*., 1969).

Feeding behaviours that have been characterized include digging, nipping, and ejecting (Foster *et al*., 1969). The dig is a behaviour in which the fish approaches the substrate, thrusts its nose into it, and then withdraws it; this is thought to be a food-seeking behaviour (Foster *et al*., 1969). The nip occurs when the fish protrudes its mouth and grasps the object from the substrate (Foster *et al*., 1969). The ejecting behaviour has been noted to take place after either a digging or nipping behaviour in which flagfish release or spit out whatever material they had just ingested from the previous behaviour (Foster *et al*., 1969).

The comfort behaviours have been defined as yawning, chafing, and gasping (Baerends and Baerends-van Roon, 1950). Yawning has been described as when the fish opens and protrudes its mouth while it has paused from swimming and its opercles are slightly flared (Foster *et al*., 1969). The chafe behaviour is believed to involve the fish "scratching" itself by rubbing itself against a hard surface (ex. air-stone) (Foster *et al*., 1969). The gasp is another behaviour in which the fish approaches the water's surface and gulps (opens and closes mouth) while continuing to swim in a forward direction (Foster *et al*., 1969).

Finally, the behaviour of fanning is hard to classify into one section because it has been associated with parental care, food-seeking, and egg cannibalism (Foster *et al*., 1969; Mertz and Barlow, 1966). The fanning display is only exhibited by males, and the male mostly remains in one place during it (Foster *et al*., 1969; Mertz and Barlow, 1966). Fanning involves vigorous beating of the caudal fin and peduncle which is compensated for by the activity of the pectoral fins (Mertz and Barlow, 1966).

2.4.4 Examples of Use & Sensitivity

There are many instances in which flagfish have been used as the test species for acute toxicity testing. Some examples involving flagfish include the impacts of pulse-exposure to methoxychlor and hydrogen cyanide on reproduction (Holdway and Dixon, 1986; Cheng and Ruby, 1982). Flagfish were used in both these instances because of their short life-cycle of approximately 90-120 days (Holdway and Dixon, 1986). Flagfish also have similar thresholds to toxicants as northern cold-water species (McKim, 1977).

Other acute and chronic toxicity studies involving flagfish have been reported. A study of 10 chlorinated organic compounds, for example, TCB, PCP, and TCE, were completed to determine a number of different LC_{50} values with regards to flagfish (Smith *et al*., 1991). This study was also completed in order to compare flagfish sensitivity to those of other commonly used native test species such as fathead minnows (Smith *et al*., 1991). This study noted that flagfish do have a similar sensitivity to chemicals as other commonly used test species, it found that they are easy to raise and can serve as a good warm-water test species, and that the more chlorinated the compound the more toxic it was (Smith *et al*., 1991). This research demonstrated flagfish sensitivity to chlorinated chemicals.

2.5 Endosulfan

2.5.1 Organochlorine Insecticides

Currently, many organochlorine insecticides have been banned for use because of their high toxicity, persistence, bioaccumulation, and ability for long-range transport

(Yao *et al*., 2008). Some of the typical characteristics of organochlorine insecticides are; their low water solubility, and high lipophilicity, and they are stable solids with limited vapour pressure (Walker *et al.*, 2006).

It is well known that once pesticides become airborne they can be dispersed by wind, deposited by wet or dry deposition, and can undergo atmospheric degradation (Shen *et al*., 2005; Tuduri *et al*., 2006). Such contaminants can also undergo long-range transport depending on their persistence in the environment (Shen *et al*., 2005; Tuduri *et al*., 2006).

Specifically, pesticides can enter aquatic ecosystems through point or non-point sources such as; direct application, surface runoff from soil and pavement, and through urban and industrial discharges leading to atmospheric deposition (Broomhall, 2002; Deger *et al*., 2003; Jergentz *et al*., 2004; Leonard *et al*., 1999, 2000, 2001; Sharma, 1990).

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxanthiepine, 3-oxide) is a broad spectrum organochlorine insecticide that was introduced in 1954 under the name Thiodan (Maier-Bode, 1968; Naqvi and Vaishnavi, 1993). The technical grade of endosulfan is composed of two isomers; α and β in a 7:3 ratio (Arnold *et al.*, 1996; Sutherland *et al*., 2004). Endosulfan is one of the few cyclodiene pesticides still used throughout the world, because it is less persistent in the environment than many of the other organochlorine insecticides (Goebel *et al*., 1982; Shen *et al*., 2005).

2.5.2 General Use - Use in Ontario

Endosulfan is currently used on both a global and local scale, most often for pest control on a variety of agricultural and horticultural crops, including; vegetables, cereals, fruits, and tobacco (Sutherland *et al*., 2004; Tuduri *et al.*, 2006).

On a global scale endosulfan has been banned in many countries including; Sweden, Colombia, Singapore, Germany, the Netherlands, and the UK (Ballesteros *et al*., 2009; Garrett, 2004). However, it is still in use in Argentina, India, Africa, Australia, Canada, and many others countries (Ballesteros *et al*., 2009; Garrett, 2004). Some of its major uses around the world are on rice fields in developing countries, cotton fields in Australia, and many other crops previously mentioned (Kennedy *et al*., 2001; Siang *et al*., 2007; Sunderam *et al*., 1992).

On a more local scale, endosulfan is the most frequently detected organochlorine pesticide across Canada, is the most heavily applied organochlorine insecticide in Ontario, and more specifically has been used extensively in the Durham region (Harris *et al*., 2000; Yao *et al*., 2008). Typically in Ontario, endosulfan is used for pest management of leafhoppers, tarnished plant bug, silver-leaf whitefly, aphids, and cyclamen mite (Harris *et al*., 2000). Recent use has been estimated at approximately 3,698 kg of endosulfan used on all surveyed crops in Ontario in 2003 (McGee *et al*., 2003). The CCME has stated that a safe level of 0.02 µg/L should be adhered to for the protection of freshwater aquatic life (CCME, 1999).

Endosulfan is readily available for application as a wettable powder or emulsifiable concentrate and the main methods of application are either through air-blast or ground boom sprayers (Garrett, 2004; Harris *et al*., 2000). Such crop applications of

endosulfan allow it to become incorporated into the environment either through drift or long-range atmospheric transport (Harris *et al*., 2000). Once in the environment it has a few different fates.

2.5.3 Endosulfan Fate in Environment

2.5.3.1 Water

The endosulfan concentrations in Ontario surface water have ranged from <0.01 mg/L to 0.54 mg/L over the past two decades (Harris *et al*., 2000). In water samples, the alpha endosulfan isomer has been found to be more persistent than the beta endosulfan isomer (Sutherland *et al*., 2004). Depending on the chemical properties of the receiving water, endosulfan can persist from 1-6 months (Naqvi and Vaishnavi, 1993).

In general, as the pH of the water increases so does the rate of endosulfan breakdown through hydrolysis (Capkin *et al*., 2006). Thus, as the pH of receiving water increases the major aquatic metabolite present is endosulfan diol, and as the pH decreases, the major aquatic metabolite present is endosulfan sulfate (Capkin *et al*., 2006; Naqvi and Vaishnavi, 1993; Sutherland *et al*., 2000). Under aerobic conditions, endosulfan is able to undergo degradation through both hydrolysis and oxidation however, under anaerobic conditions, it can only undergo hydrolysis (Figure 1) (ASTDR, 2000).

2.5.3.2 Soil

In soil samples the beta endosulfan isomer has been found to be more persistent than the alpha endosulfan isomer and the half-life of endosulfan in soil has been shown to range anywhere from a few months to over 2 years (Harris *et al*., 2000; Naqvi and Vaishnavi, 1993; Sutherland *et al*., 2004). The primary route of degradation in the soil is via microbial biotransformation, and the major metabolite produced is endosulfan sulfate (Figure 1) (Harris *et al*., 2000).

2.5.3.3 Air

As mentioned previously, the application of endosulfan to crops can lead to long-range atmospheric transport (Harris *et al*., 2000). A specific example of long-range transport was the detection of endosulfan and endosulfan sulfate in arctic samples where it has never been used (Garbarine *et al*., 2002; Harris *et al*., 2000; Hung *et al*., 2002). From air, endosulfan can be deposited into water or soil to be further broken down, or can be degraded in the atmosphere into endosulfan diol and endosulfan α-hydroxyether through photolysis (Figure 1) (ASTDR, 2000).

2.5.4 Metabolites / Degradation Pathway

As mentioned previously, endosulfan can be degraded into many different products including; endosulfandiol, endosulfan sulfate, endosulfan ether, hydroxyendosulfan ether, and endosulfan lactone (Goebel *et al.*, 1982; Gupta and Gupta, 1979). However, the two major metabolites most often observed in the environment are

endosulfan diol and endosulfan sulfate (Figure 1) (ASTDR, 2000; Peterson and Batley, 1993; Sutherland *et al*., 2004).

Endosulfan diol is the result of endosulfan undergoing a hydrolysis reaction, and endosulfan sulfate is the result of an oxidation reaction (Garrett, 2004; Naqvi and Vaishnavi, 1993). Once endosulfan is metabolized into endosulfan sulfate, it can be transformed into endosulfan lactone, and then either of the major metabolites can be further transformed into endosulfan ether or endosulfan hydroxyether (ASTDR, 2000). Since, the major metabolite of endosulfan has been found to be endosulfan sulfate, which is highly toxic to fish, persistent and bioaccumulative, the biotransformation of endosulfan to endosulfan sulfate is not considered a detoxification process (Rao *et al.*, 1981) (Figure 1).

2.5.5 Endosulfan Mode of Action

Endosulfan is believed to act directly on the central nervous system of fish (ASTDR, 2000; Harris *et al*., 2000). The proposed mechanism of endosulfan is that it binds to the picrotoxin site in the gama-aminobutyric acid (GABA) chloride ionophore complex (Coats, 1990; Harris *et al*., 2000). Normally, GABA is an inhibitory neurotransmitter which binds to the GABA-gated chloride channel and allows an increased flow of chloride into the nerve cells (Bradbury *et al*., 2008; Coats, 1990; Woolley, 1995). This increased flow causes neuronal hyperpolarization and depresses excitability (Bradbury *et al*., 2008). In this proposed mechanism, when endosulfan binds to the picrotoxin site it impairs the inhibitory function of the normal GABA channel,

thereby impairing the chloride flux, which results in hyperexcitation and possibly convulsions (Bradbury *et al*., 2008; Coats, 1990; Harris *et al*., 2000).

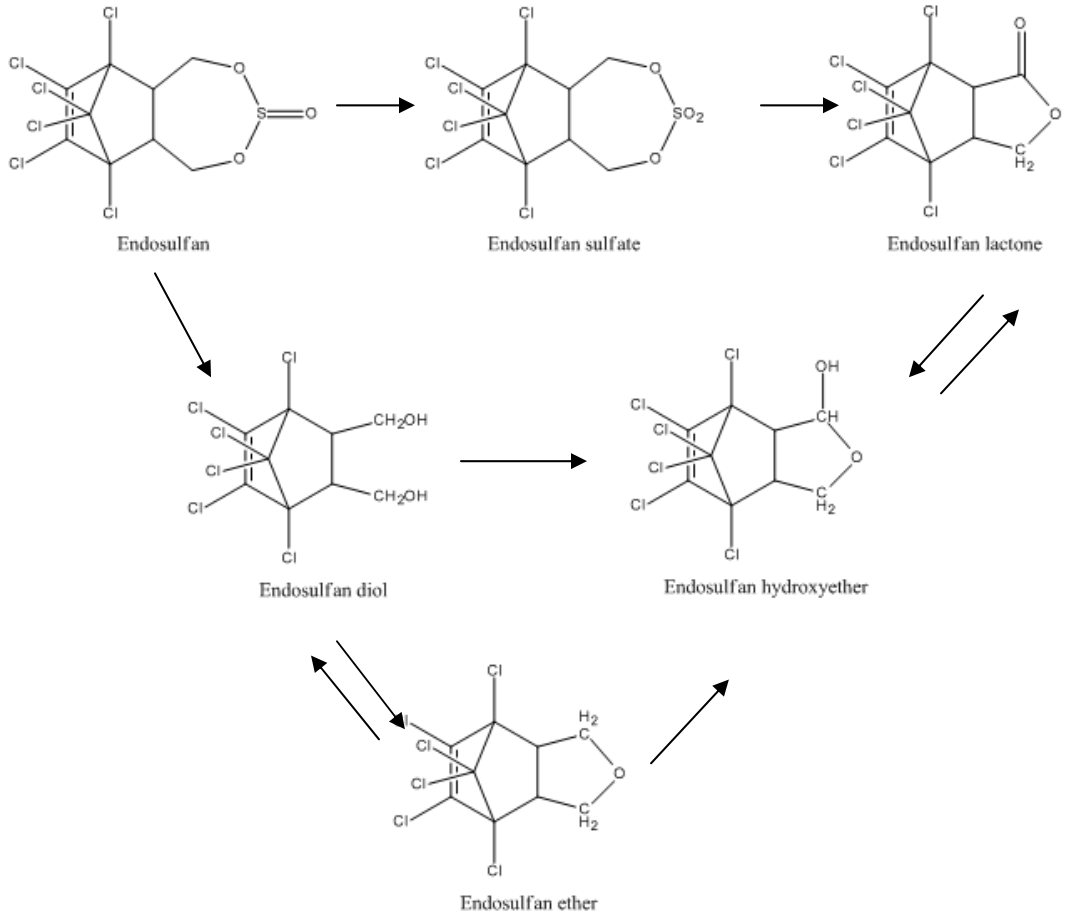
There is an alternate hypothesis for the toxic mechanism of action of endosulfan in which it is postulated that alpha endosulfan inhibits $Ca^{2+}Mg^{2+}$-ATPase and Ca-ATPase; however it is not as widely accepted as the GABA explanation (ASTDR, 2000; Coats, 1990; Harris *et al*., 2000).

2.5.6 Endocrine Disruption

Endocrine disruptors have been defined as synthethic or naturally occurring chemicals that mimic naturally occurring hormones and alter the endocrine system (Guillette and Gunderson, 2001; Jobling *et al*., 1998). In wildlife, there have been many chemicals that have been documented with disrupting and altering reproductive, morphological, and physiological development (Guillette and Gunderson, 2001). Endocrine disrupting chemicals can cause effects via different modes of action (Kime *et al*., 1999). Specifically, in fish, xenobiotics can act on the hypothalamic-pituitary-gonadal axis, or they can act directly on the liver (Kime *et al*., 1999). They can alter the hormone production and release at both the hypothalamus and pituitary, altering the gonadotrophin releasing hormone, and the gonadotrophins, respectively (Appendix 1) (Arcand-Hoy and Benson, 1998; Kime *et al*., 1999). When acting directly at the liver it affects the production of vitellogenin, an egg yolk precursor protein (Appendix 1) (Arukwe, 2001; Kime *et al*., 1999). Alterations in any of the endocrine system can lead to effects in sexual differentiation and gamete development (Arukwe, 2001; Guillette and

Gunderson, 2001). Since endosulfan is a potential endocrine disruptor it is postulated to be acting on one of these possible mechanisms however, the exact mechanism and location is still to be elucidated.

Figure 1: The metabolic pathways for endosulfan showing the possible metabolites. The major metabolites created are endosulfan sulfate through an oxidation reaction, and endosulfan diol through a hydrolysis reaction. This figure was created using ChemBioDraw Ultra 11.0 and is adapted from the ASTDR, 2000.



2.6 Toxicity of Endosulfan to Fish

The following provides a brief overview of some of the toxicity effects that have been observed in fish due to acute or chronic exposure to endosulfan.

2.6.1 Acute Toxicity

There is a significant amount of information known about the acute effects of endosulfan on aquatic organisms (Harris *et al*., 2000). Endosulfan is highly toxic to fish with LC_{50} values reported in the parts per billion, and it tends to accumulate in their fatty tissues (Ballesteros *et al*., 2007; EPA, 1980; Naqvi and Vaishnavi, 1993; Jonsson and Toledo, 1993). Acute effects of endosulfan on fish are due to direct neurological interference, and are often manifested by hyperactivity and erratic swimming, followed by a loss of equilibrium, muscle tremors, and then convulsions or immobility prior to death (ASTDR, 2000; Gopal *et al*., 1981; Harris *et al*., 2000). Liver and kidney are the main sites of detoxification for endosulfan (Rao *et al*., 1981).

Acute 96-h LC_{50} values for various species of fish range from as low as 0.02 µg/L to 14 µg/L (Sunderam *et al*., 1992), and include; 0.02 µg/L for the harlequin fish (*Rasbora heteromorpha*), 2.2 µg/L for firetail gudgeon (*Hypseleotris gallii*), 3.1 µg/L for mosquitofish (*Gambusia affinis*), 0.42 µg/L for Asian swamp eels (*Monopterus albus*, Zuiew), 0.6 µg/L for European carp (*Cyprinus carpio*), 5.9 µg/L for eastern rainbow fish (*Melanotaenia duboulayi*), 5.7 µg/L silver perch (*Bidyanus bidyanus*), 1.3 µg/L for bony bream (*Nematolosa erebi*), 1.2 µg/L for golden perch (*Macquaria ambigua*), and 1.6 µg/L for rainbow trout (*Oncorhynchus mykiss*) (Alabaster, 1969; Gopal *et al*., 1981;

Siang *et al*., 2007; Sunderam *et al*., 1992). However, choice of test method and life-stage can lead to a wide variation in acute toxicity (Sunderam *et al*., 1992).

A recent endosulfan study by Ballesteros *et al* (2007) determined the LC_{50} value for Rio De La Plata Onesided Livebearer (*Jenynsia multidentata)* and evaluated the histological effects on gills and liver. An LC_{50} value of 0.719 µg/L for males and 1.317 µg/L for females was determined; the difference between males and females was presumably due to a higher lipid content in females (Ballesteros *et al*., 2007). Effects of endosulfan on the gills included hypertrophy and lifting of the epithelium; liver effects were concentration dependent, with the highest concentration causing necrosis (Ballesteros *et al*., 2007). Similar effects of endosulfan on the gills and livers were also observed in rainbow trout (Capkin *et al*., 2006).

2.6.2 Chronic Toxicity

There have been fewer chronic studies of endosulfan on aquatic organisms because of the cost and length of time required. Observed chronic effects include primary nervous and respiratory system responses, and secondary effects on the respiratory, digestive (hepatic) and reproductive systems (Harris *et al*., 2000). Zebra fish (*Brachydanio rerio*) exposed to 0.4 µg/L of endosulfan for 21-d had significant histopathological alterations including necrosis in the gill lamella, and zonal necrosis in the liver along with lipid accumulation (Toledo and Jonsson, 1992).

Reproductive effects of endosulfan have included reduced ovarian activity, an increase in immature and atretic oocytes, a thicker ovarian wall, enhanced amount of oogonia in stage I, reduced diameter of oogonia in stage II and III oocytes, and decreased

yolk-vesicle and yolk accumulation in stage II and III oocytes (Pandey, 1988; Shukla and Pandey, 1986). Also, a multi-generation assessment of a 4-h pulse-exposure of endosulfan to crimson-spotted rainbow fish (*Melanotaenia fluviatilis*) of concentrations ranging from 1.0 to 50 µg/L reported a decreased rate of hatch in eggs collected from exposed adults (Holdway *et al*., 2008).

2.7 Rationale

This research is necessary in order to better characterize the effects that endosulfan may be having on non target organisms, such as fish. Using both acute and chronic studies the effects of endosulfan on growth and survivability will be assessed. Since endosulfan is classed as a potential endocrine disruptor it is also of importance to better characterize its potential reproductive effects. These acute and chronic measures of toxicity will help to elucidate what effects, if any, may be present, and how they may manifest themselves within the fish population.

3.0 Materials and Methods

3.1 Laboratory Fish

First generation laboratory raised flagfish were maintained, and their second generation offspring were used in all of the experiments. The initial stock populations were housed in 70 L glass flow-through tanks which were equipped with an air-stone and greenery. All experiments were on a 16-h light: 8-h dark schedule, with 0.5 h of dawn and dusk each included in the light cycle.

3.2 Chemicals

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxanthiepine, 3-oxide), analytical standard ($\alpha + \beta \sim 2 + 1$), was purchased from Sigma-Aldrich Co. and was made into a set of stock solutions. Analytical grade acetone (>99%) was used as the solvent (carrier) for all experiments and was also purchased from Sigma-Aldrich. Endosulfan solutions were prepared by weighing out the appropriate amount of endosulfan and dissolving it in acetone, serial dilutions were completed to reach the desired concentrations. In all experiments acetone never exceeded 20 µl/L of dilution water and was always equal in all treatments.

3.3 Feed

Three types of fish feed were used throughout the experiments which included flake food, frozen brine, and fresh brine shrimp. The Tetramin Pro flake was obtained from Big Al's (Oshawa, Ontario) and is a product of Tetra, a Spectrum Brand Company.

It had a minimum crude protein of 46.0%, 12.0% crude fat, 3.0% crude fibre, 1.1% phosphorus, 200 mg/kg ascorbic acid, and maximum moisture of 8.0%. The Bio-pure frozen brine shrimp was purchased at Big Al's and is a product of Hikari. The Bio-pure frozen brine shrimp is composed of 6.8% crude protein, 1.5% crude fat, 1.2% crude fibre, and has a maximum of 86.0% moisture. Finally, the brine eggs for the brine shrimp nauplii were premium grade brine shrimp eggs and were bought from Brine Shrimp Direct.

3.4 Water Parameters

3.4.1 Nitrate, Nitrite, Chlorine, Alkalinity, Hardness

A Quick Dip 6-n-1 test strip produced by Jungle Laboratories Corporation was used to measure the nitrate, nitrite, total hardness, total alkalinity, total chlorine, and pH of the lab water. The strip was dipped into the water and removed immediately and held level for 30 seconds before reading the hardness, alkalinity, chlorine, and nitrate by comparing it to a freshwater colour chart. After 60 seconds had elapsed, the nitrite was also compared to the colour chart.

3.4.2 Dissolved Oxygen

A LaMotte dissolved oxygen water quality test kit was used to monitor the level of dissolved oxygen in lab water. The first step of the kit was to collect the water sample, and then add 8 drops of manganous sulphate solution, followed by 8 drops of alkaline potassium iodide azide solution. The mixture was then capped and mixed gently. A

precipitate was formed and was allowed to settle to just below the shoulder of the bottle. Next, 8 drops of sulphuric acid, 1:1 was added to the bottle and once again another precipitate was formed. The bottle was gently inverted and mixed until the precipitate completely dissolved; at this point the sample was fixed and could be stored until the rest of the protocol could be completed. Due to the fact that sampling was being conducted in the lab it was not necessary to store samples. Instead the protocol was completed from beginning to end all at once. The next step was to transfer 20 mL of the sample into the test tube. The direct reading titrator was then filled with sodium thiosulfate and was titrated into the test tube until a pale yellow colour was reached. Once the yellow colour was seen, 8 drops of starch indicator was added to the test tube. The titration continued until the blue colour disappeared and the solution was colourless. Using the direct reading titrator the amount of dissolved oxygen in parts per million (ppm) could be read directly off of it.

3.4.3 Temperature, pH, and Conductivity

A waterproof combo pH, EC/TDS, and temperature meter was purchased from Hanna Instruments, Laval, Quebec and was used for daily measurements. The meter was calibrated for two points (pH 7.0 and pH 10.0) daily, before measurements were taken and the conductivity was calibrated to one point (1314 μS/cm) daily.

3.5 Breeding Tank Set-up

Stocks of flagfish were maintained in the lab as per section 3.1, and were then selected for use in breeding tanks. The breeding groups were made up of 4 females and 2 males. The fish were selected so that one dominant male (largest in size) and one sub-dominant male were present; females that were slightly smaller than the males were chosen. The breeding groups were set-up in 70 L glass flow-through tanks that contained an air-stone, greenery, and one breeding substrate. The breeding substrate was made out of a piece of glass with well washed green yarn wrapped around it.

3.6 Egg Collection

Breeding substrates were removed daily and rubbed down to dislodge any eggs into a white plastic collecting container. The substrate was then rinsed and replaced back into the tank. The collection containers were immediately put into the warm room and the eggs in each container were enumerated. As each egg was being counted it was removed from the collection dish and placed into a plastic petri dish which contained rearing solution. There was a maximum of 100 eggs per dish. The rearing solution is a mixture of 10% NaCl, 0.30% KCl, 0.40% $CaCl_2 \cdot 2H_20$, 1.63% $MgSO_4 \cdot 7H_20$, 0.01% methylene blue, and distilled water.

3.7 Larval Rearing

Once flagfish hatched and absorbed their yolk sac they were transferred to 300 mL crystallization dishes containing 200 mL of water. Fish were fed freshly hatched

brine shrimp, dishes were siphoned, and new water was added daily. Fish were transferred to aquaria as soon as they were large enough and were maintained there.

3.8 Glassware Cleaning

All glassware was cleaned using a soak for 24 hours in Contrad 70 solution, followed by a thorough wash on the organic chemical cycle in the dishwasher.

3.9 Water Analysis

Actual endosulfan water concentrations were determined from a 4 mL water sample that was taken during the 4 hours pulse-exposure full life-cycle study (Appendix 2). The water samples were stored in -80 ºC until they were taken to an external lab for analysis. The samples were analyzed via gas chromatography / electron capture detection (GC/ECD) at the York-Durham Regional Environmental Laboratory, Pickering, Ontario. The nominal values were used for data interpretation of the full life-cycle study.

3.10 Statistical Analysis

Data was analyzed with STATISTICA 8.0 software package. Data was checked for normality using Shapiro-Wilk's W test for normaility, and Brown and Forsythe's test for homogeneity of variances ($p \leq 0.05$). Data was transformed when results were not homogenous. One-way and factorial analysis of variance (ANOVA) was used to analyze overall differences among treatments. Where significant differences ($p \leq 0.10$) were seen Tukey's HSD post-hoc test gave more detailed information on differences. A larger type

I error (α) of 0.1 was chosen in order to reduce the type II error (β) (false negative) and thus increase power (1-β).

3.11 Experiment #1: 96-h Continuous Exposure LC_{50} Study

Eggs were collected and reared as described in section 3.6 and 3.7. 2-d-old larval fish were continuously exposed to nominal concentrations of endosulfan 0 (control), 0 (acetone control), 0.01, 0.03, 0.10, 0.32, 1.0, 3.2, 10, and 32 µg/L. The fish were starved throughout the experiment. A 95% static renewal of the endosulfan solutions were completed every 24 hours until the 96 hours had elapsed. The water parameters discussed in section 3.4 were monitored daily. The fish were monitored for mortality at 30, 60, 90, and 120 minutes, and 4, 6, 8, 12, 16, 24, 48, 72, and 96 hours.

3.12 Experiment #2: 2, 4, 8-h Pulse-exposure Study

Eggs were collected and reared as described in section 3.6 and 3.7. All 2-3 d-old post-hatch larvae were pooled and pulse-exposed to nominal concentrations of endosulfan 0 (control), 0 (acetone control), 1.0, 3.2, 10, 32, and 100 µg/L for either 2, 4, or 8-h. The fish were then removed to freshwater where they were held for the duration of the experiment (96 hours); the fish were also starved during the experiment.

The water in the crystallization dishes containing the larval fish was renewed every 24 hours and the water parameters were measured daily as per section 3.4. The fish were monitored at 2, 4, 6, 8, 12, 16, 24, 48, 72, and 96 hours for mortality and any behavioural changes were noted.

3.13 Experiment #3: 4-h Pulse-exposure LC_{50} Study

Eggs were collected and reared as described in section 3.6 and 3.7. Selected 3-d-old post-hatch larvae were pulse-exposed to nominal concentrations of endosulfan 0 (control), 0 (acetone control), 32, 56, 100, 140, and 200 µg/L. The fish were removed to freshwater and remained there for the duration of the experiment. Water was renewed every 24 hours and was monitored daily for the water parameters as described in section 3.4. Test fish were starved throughout the experiment and were monitored at 4, 8, 12, 16, 24, 48, 72, and 96 hours for mortality and behavioural changes.

3.14 Experiment #4: 4-h Pulse-exposure Sub-lethal Study

Eggs were collected and reared as described in section 3.6 and 3.7. 4-5 d-old post-hatch larvae were pulse-exposed for 4 hours to nominal concentrations of endosulfan 0 (control), 0 (acetone control), 0.32, 1.0, 3.2, 10, 32 µg/L. After the pulse-exposure larval fish were removed to freshwater which was renewed every 24 hours and monitored daily for water parameters described in section 3.4. The fish were starved throughout the experiment. The fish were also monitored at 0, 4, 8, 12, 16, 24, 48, 72, and 96 hours for mortality, loss of equilibrium, axis malformation and were ranked for health. At 2, 4, 8, 16, 24, 30, 48, 54, 72, 78, and 96 hours photographs of the fish were taken with a Leica microscope.

3.15 Experiment #5: Full Life-Cycle Study

3.15.1 Egg Collection

Eggs were collected daily from stock tanks which were composed of 2 male and 4 female second generation laboratory raised fish. All flagfish used in this study were obtained from two batches of eggs collected from the stock tanks, and 1-2 d-old post hatch larvae were pooled. Larval flagfish were fed brine shrimp nauplii, and uneaten food and faeces was removed daily. Test fish were not fed 24 hours prior to being moved into crystallization dishes or aquaria.

3.15.2 Pulse-exposure and First Growth Measurement

On day zero fish were randomly selected from the pooled batch and thirty larval flagfish and were placed into each of the crystallization dishes using an adapted transfer pipette. The experiment was run in duplicate with 30 fish per dish and one extra spare of 30 control fish was also run simultaneously. The endosulfan stocks and acetone were added using a calibrated micropipettor to each of the crystallization dishes which already contained the fish and the appropriate amount of lab water. The fish were pulse-exposed for 4 hours to nominal endosulfan concentrations of 0 (control water), 0 (acetone control), 0.10, 0.32, 1.0, 3.2, or 10 μg/L. The final volume of each crystallization dish was 200 mL. During the pulse-exposure a 4 mL water sample was removed from each dish in order to be analyzed for actual endosulfan concentrations. Fish were left for the 4-h pulse-exposure time period and were then transferred into new crystallization dishes with fresh lab water.

Photos of the larval flagfish were taken by placing the crystallization dishes on top of a 1 mm grid. These photos were taken so that a day zero measurement of their size could be recorded. After the pulse-exposure, fish were reared in the crystallization dishes being fed brine shrimp nauplii once daily. Excess food and waste was siphoned and water was renewed daily in the crystallization dishes. Water parameters were also checked daily as per section 3.4 and mortality and deformity observations were noted.

3.15.3 Transfer to 10 L Aquaria and Second Growth Measurement

Fish were moved into 10 L flow-through aquaria from crystallization dishes. Before being moved, photos of the larval flagfish were taken again using the 1 mm grid. The tanks were set-up with a flow rate (0.694 mL/sec) that allowed 6 complete water changes in every 24 hour time period. The fish were fed three times daily while in 10 L tanks. They were fed a mixture of flake food and freshly hatched brine shrimp. The tanks were siphoned daily and the water parameters were monitored daily along with mortality.

3.15.4 Transfer to 70 L Aquaria

Fish were again transferred into 70 L flow-through aquaria. Each tank had a breeding substrate added into the tank and they were watched for guarding behaviour. The fish were fed 5 times daily with a mixture of flake food, freshly hatched brine shrimp, and frozen brine shrimp. Uneaten food and faeces were removed daily from the tanks. The flow rate (5.14 mL/sec) was set accordingly so that 6 water changes occurred

in every 24 hour time period and the water parameters in the tanks were monitored daily, along with mortality.

3.15.5 Thinning, Egg Collection, and Larval Rearing

Fish were separated into their breeding harem, 2 males and 4 females per tank, the excess fish were thinned into separate 70 L tanks for holding until dissections could be completed. The fish continued to be fed 5 times daily with the same mixture as listed above, and tanks continued to be siphoned daily. Once the breeding tanks were set-up the substrates were checked daily for eggs as described in section 3.6. The eggs were only kept if more than 30 were present; otherwise the eggs were counted and discarded.

Once a state of steady spawning, defined as 4 days in a row of greater than 30 eggs, had been reached the eggs were once again discarded after being enumerated. A minimum of 5 egg collections, of greater than 30 eggs, were made and eggs were monitored daily for fungus and hatchability. Any eggs containing fungus were enumerated and removed from the petri dishes and the dishes were checked daily. Of the eggs collected, 30 larval fish from each of the treatments were kept and raised for 30 days post-hatch. All other larval fish were euthanized and discarded. Once the 30 fish had absorbed the yolk sacs they were started on a diet of brine shrimp nauplii 3 times per day. At day 30 post-hatch, the larval fish were euthanized and length and weight measurements were taken. For a more detailed timeline of events for the full life-cycle study refer to Appendix 3.

3.15.6 Dissections

The fish that were thinned were euthanized using a concentration of 250 mg/L of MS-222. The fish were sexed, and length and weight was taken using a calliper and analytical balance. After growth measurements were completed the fish were dissected and the liver and gonads were removed. The gonads and liver were weighed using an analytical balance and a calculation of gonadosomatic index, hepatosomatic index, and condition factor were completed with the data obtained.

4.0 Results

4.1 96-h Continuous exposure LC_{50} Study

4.1.1 Abiotic Factors

There were no significant differences in temperature, pH, dissolved oxygen, alkalinity, and hardness over the duration of the 96-h continuous exposure experiment (Table 1).

4.1.2 Toxicity Values

There were no significant differences between replicates in the 96-h continuous exposure data so the data were pooled. The 96-h continuous exposure LC_{50} for larval flagfish was 4.35 µg/L based on cumulative percent mortality (Figure 2).

A static renewal continuous exposure experiment using concentrations of 0.01 µg/L, 0.03 µg/L, 0.10 µg/L, 0.32 µg/L, 1.0 µg/L and 3.2 µg/L of endosulfan had no significant difference in mortality relative to the controls (Figure 3). There was, however, 30% mortality observed in larval flagfish exposed to 3.2 µg/L, and non-lethal effects such as hyperactivity and convulsions were also noted (Figure 3). Mortality observed in the 10 µg/L and 32 µg/L treatments was significantly different than the controls post 48-h exposure. The LOEC for a 96-h continuous exposure of endosulfan to larval flagfish was 10 µg/L and the NOEC was 3.2 µg/L.

Table 1: Abiotic factors measured on lab water collected through-out each of the experiments. Values are written as means ± standard error. *Not Measured because instrument was not yet available for use.

Experiment	Parameter	Mean ± Standard Error
Experiment #1: 96-h continuous exposure	Temperature (ºC)	24.6 ± 0.04
	pH	7.43 ± 0.01
	Dissolved Oxygen (ppm)	7.90 ± 0.03
	Alkalinity (ppm as $CaC0_3$)	0.00
	Hardness (ppm as $CaC0_3$)	0.00
	Conductivity (µS/cm)	NM*
Experiment #2: 2, 4, 8-h pulse-exposure	Temperature (ºC)	23.3 ± 0.05
	pH	7.47 ± 0.02
	Conductivity (µS/cm)	28.0 ± 1.01
	Dissolved Oxygen (ppm)	7.90 ± 0.03
	Alkalinity (ppm as $CaC0_3$)	0.00
	Hardness (ppm as $CaC0_3$)	0.00
Experiment #3: 4-h pulse-exposure	Temperature (ºC)	22.6 ± 0.05
	pH	7.26 ± 0.01
	Conductivity (µS/cm)	45.0 ± 0.48
	Dissolved Oxygen (ppm)	7.80 ± 0.00
	Alkalinity (ppm as $CaC0_3$)	0.00
	Hardness (ppm as $CaC0_3$)	0.00
Experiment #4: 4-h sub-lethal pulse-exposure	Temperature (ºC)	22.8 ± 0.04
	pH	7.32 ± 0.01
	Conductivity (µS/cm)	72.8 ± 1.37
	Dissolved Oxygen (ppm)	7.80 ± 0.00
	Alkalinity (ppm as $CaC0_3$)	0.00
	Hardness (ppm as $CaC0_3$)	0.00

Figure 2: Cumulative mortality ± standard error of larval flagfish (pooled n=20) from a continuous exposure static renewal of varying concentrations of endosulfan at 96-h. Concentrations used were 0 (control and control carrier), 0.01, 0.03, 0.10, 0.32, 1.0 3.2 10, 32 µg/L.

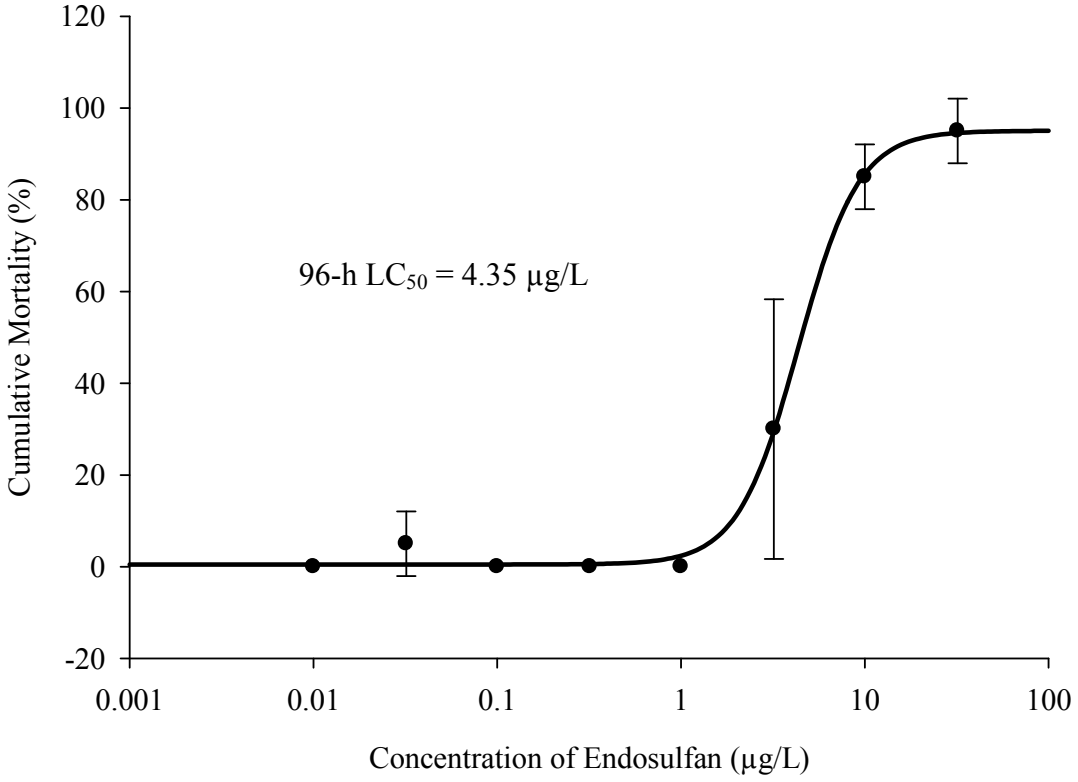
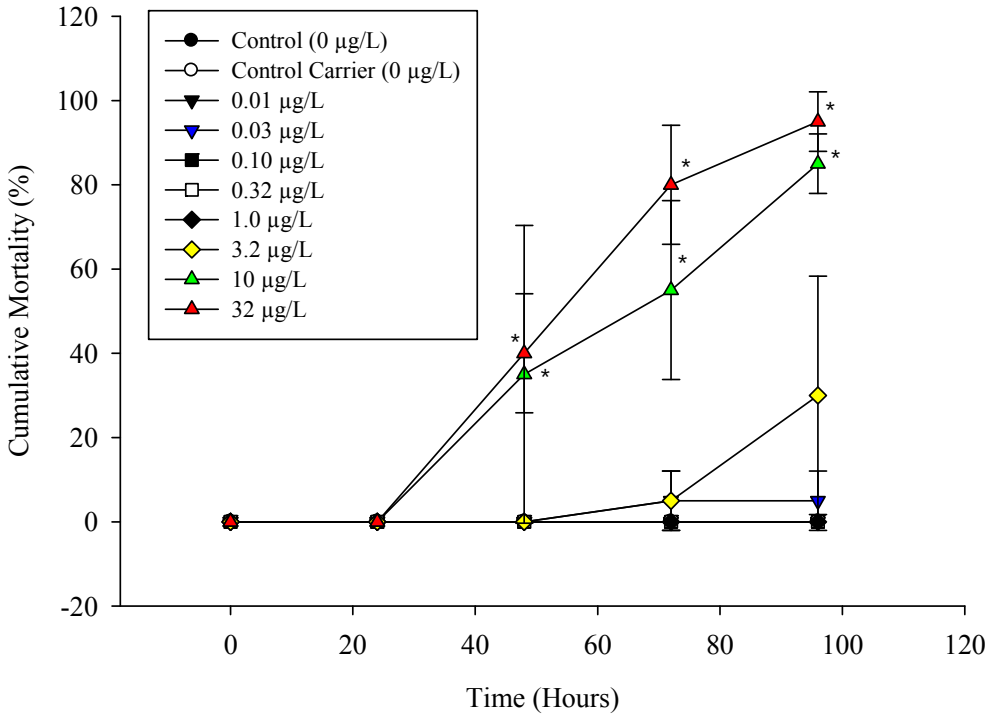


Figure 3: Cumulative percent mortality ± standard error over a 96-h continuous exposure of endosulfan to larval flagfish (pooled n=20). Significant difference from the controls is noted with an asterisk (*) $p \leq 0.10$.



4.2 2, 4, 8-h Pulse-exposure Study

4.2.1 Abiotic Factors

There were no significant differences in temperature, pH, dissolved oxygen, alkalinity, and hardness over the duration of the 2, 4, and 8-h pulse-exposure experiment (Table 1).

4.2.2 Mortality

In a range-finding experiment, larval flagfish were pulse-exposed for different durations (2, 4, or 8 h) to varying concentrations of endosulfan. There were no replicates for each of the pulse-exposures. When larval flagfish were pulse-exposed to a concentration of 100 µg/L it was observed that as the length of the pulse was increased so did the percent mortality (Figure 4).

4.3 4-h Pulse-exposure LC_{50} Study

4.3.1 Abiotic Factors

There were no significant differences in temperature, pH, dissolved oxygen, alkalinity, and hardness over the duration of the 4-h pulse-exposure experiment (Table 1).

4.3.2 Toxicity Values

The percent cumulative mortality following a 4-h pulse-exposure of endosulfan to larval flagfish for varying concentrations at 168-h gave an LC_{50} of 49.7 µg/L (Figure 5). There were no significant differences between replicates so the data was pooled for analysis, and the percent mortality increased as the exposure concentrations increased (Figure 5).

After 48-h, 100 µg/L, 140 µg/L, and 200 µg/L treatments showed significant differences in the percent mortality from the controls (Figure 6). The LOEC at 168-h for a 4-h pulse-exposure of endosulfan to larval flagfish was 32 µg/L. The NOEC was < 32 µg/L because all test concentrations showed significant effects relative to the controls.

Figure 4: Mortality of larval flagfish pulse-exposed for 2-h, 4-h, or 8-h to varying concentrations of endosulfan at 96-h (n=10). Concentrations used were 0 (control and control carrier), 1.0, 3.2, 10, 32, and 100 µg/L.

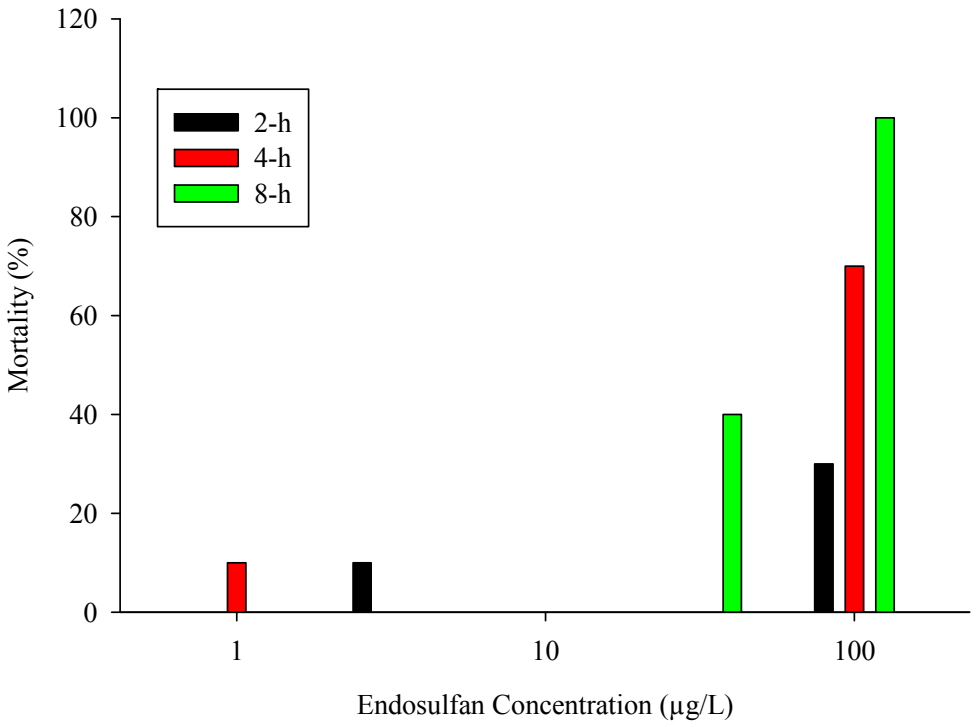


Figure 5: Cumulative mortality ± standard error of larval flagfish (pooled n=30) from a 4-h pulse-exposure of varying concentrations of endosulfan at 168-h. Concentrations used were 0 (control and control carrier), 32, 56, 100, 140, 200 µg/L.

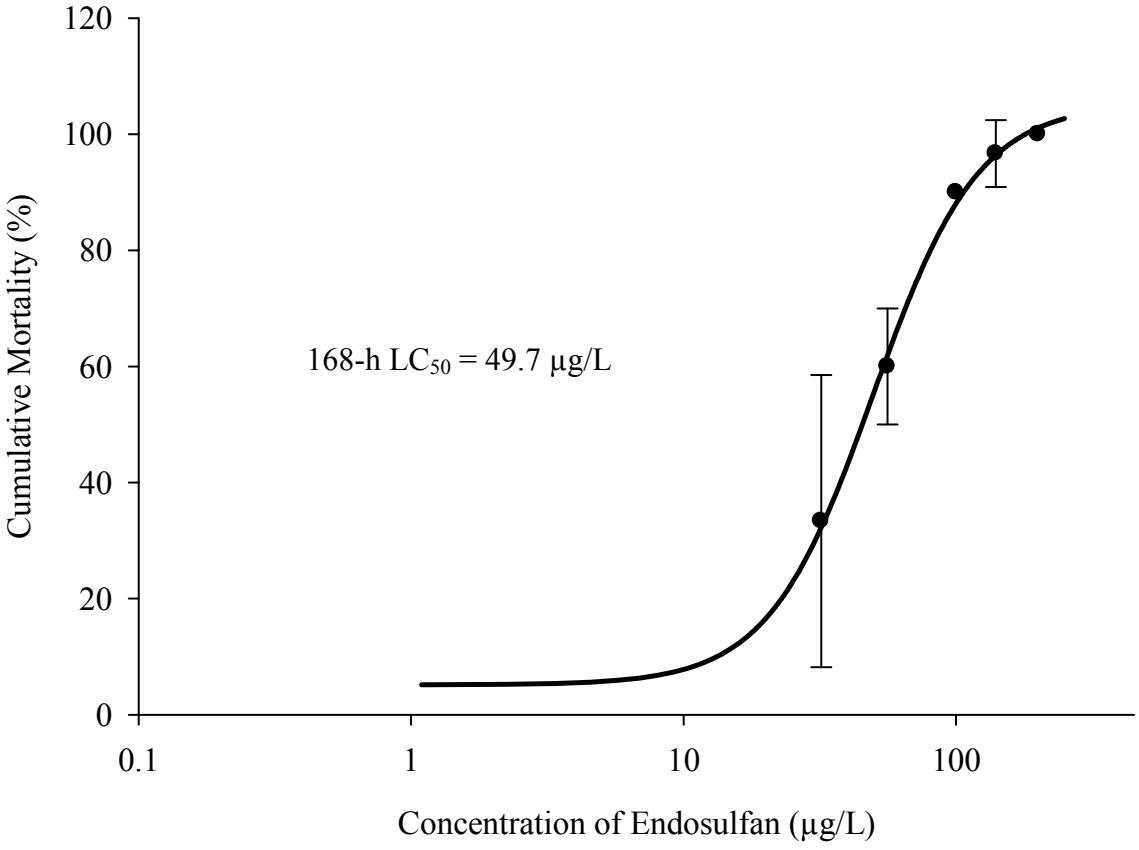
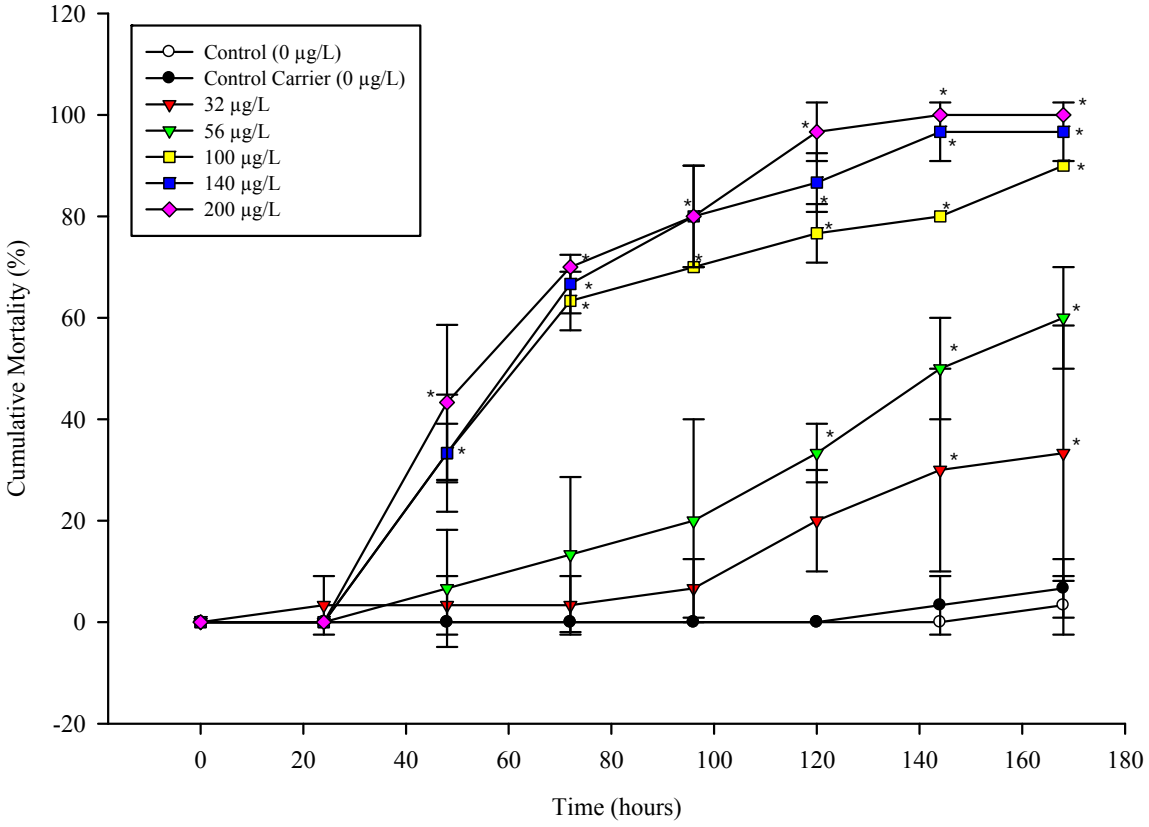


Figure 6: Cumulative percent mortality ± standard error over a 168-h 4-h pulse-exposure of endosulfan to larval flagfish (pooled n=30). Significant difference from the controls are noted with an asterisk (*) $p \leq 0.10$.



4.4 4-h Pulse-exposure Sub-lethal Study

4.4.1 Abiotic Factors

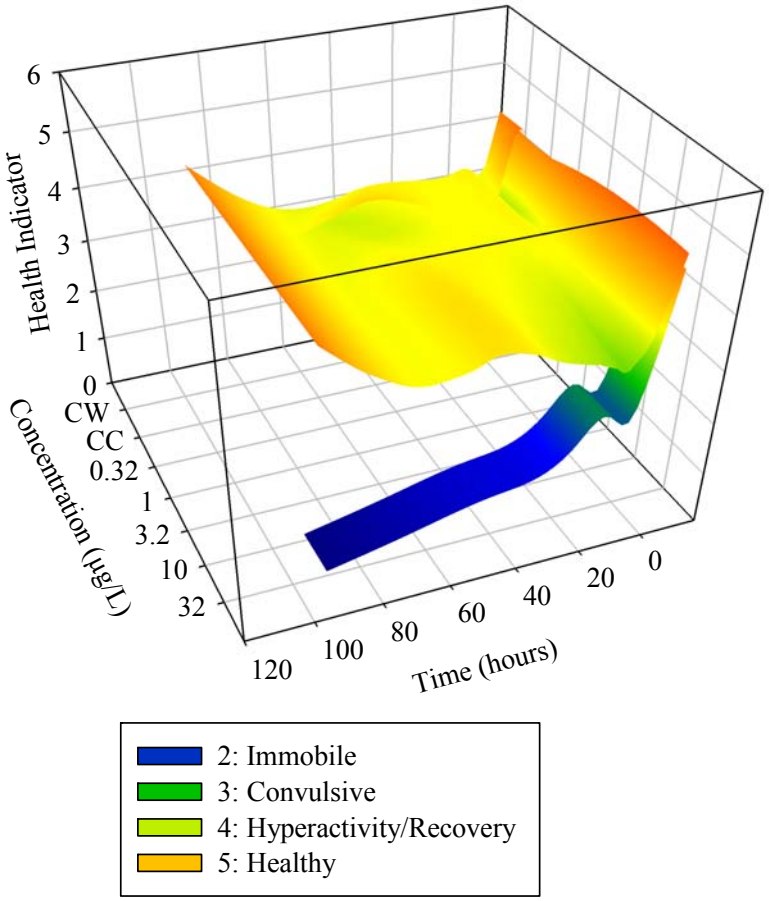
There were no significant differences in temperature, pH, dissolved oxygen, alkalinity, and hardness over the duration of the 4-h pulse-exposure sub-lethal experiment (Table 1).

4.4.2 Sub-lethal Effects

The sub-lethal effects of a 4-h endosulfan pulse-exposure of 0.32 µg/L, 1.0 µg/L, 3.2 µg/L, 10 µg/L, and 32 µg/L to larval flagfish were observed using microscopy and videos. At 96-h a slight axis malformation (bent spine) was observed in the 10 µg/L treatment while in the 32 µg/L treatment a definite axis malformation was present (Figure 7). Other sub-lethal effects observed prior to axis malformation were hyperactivity, convulsions, and immobility. Larval flagfish exposed to 0.32 µg/L, 1.0 µg/L, 3.2 µg/L, and 10 µg/L endosulfan exhibited sub-lethal effects between 24 and 48 h but were completely recovered by the end of the 96-h experiment (Figure 8). Flagfish exposed to 32 µg/L were observed to undergo hyperactivity and convulsions followed by immobilization and axis malformation; they never recovered and in some cases mortality was the final endpoint (Figure 8).

Figure 7: Photographs showing the sub-lethal effects of a 4-h pulse-exposure of endosulfan to larval flagfish at 96-h. Photographs were taken while fish were moving. Arrow indicates signs of axis malformation (bent spine). Malformations are due to hyperactivity and convulsions.

Figure 8: Cumulative health ranking of the sub-lethal effects of a 4-h pulse-exposure of endosulfan to larval flagfish over a 96-h time span (pooled n=30). The ranking indicates; 5: Healthy, 4: Hyperactivity/Recovery, 3: Convulsion, 2: Immobile, and 1: Axis Malformation. Note that even though axis malformations were visible in some treatments, as a cumulative effect they were not prevalent.



4.5 Full Life-cycle Study

4.5.1 Abiotic Factors

There were no significant differences in temperature, pH, dissolved oxygen, alkalinity, and hardness over the duration of the 4-h pulse-exposure full life-cycle experiment (Table 2).

4.5.2 Mortality

A 4-h pulse-exposure of endosulfan led to a significantly higher percentage of mortality in the 10 µg/L treatment compared to all other treatments (Table 3).

4.5.3 Growth

There were no significant differences between replicates for total length data at day zero and thus the data were pooled for interpretation. At day zero of the full life-cycle experiment in 7-8-d-old larval flagfish were slightly longer in the 0.10 µg/L treatment and compared to the 1.0 µg/L treatment with a difference of approximately 0.40 mm (Table 4).

At the second measurement of total length 49 d after the 4-h pulse-exposure there was no significant difference between the replicates so the data were pooled. The 56-57-d-old flagfish exposed to 1.0 µg/L were significantly smaller than the controls, and those exposed to 0.10 µg/L (Table 5).

Wet weight and total length data of thinned 135-136-d-old flagfish were analyzed when the breeding harems were set-up. There were no significant differences between treatment replicates so the data were pooled for interpretation. Both the control water and 10 µg/L treatments had larger total length and wet weights compared to the control carrier, 0.10 µg/L, 0.32 µg/L, and 3.2 µg/L treatments (Table 6).

There were no differences in wet weights or total length of 175-176-d-old flagfish exposed to any endosulfan treatment relative to controls (Table 7).

Replicate wet weights and total lengths of 30 day old second generation larvae could not be pooled due to significant differences, and the weight data were log transformed. Wet weights of the carrier controls and 0.10 µg/L treatments were significantly smaller than the 0.32 µg/L, 1.0 µg/L, 3.2 µg/L, and 10 µg/L treatments in replicate A (Table 8). In replicate B both the water controls and 0.32 µg/L were significantly smaller than the carrier control, 0.10 µg/L, 1.0 µg/L, 3.2 µg/L, and 10 µg/L treatments (Table 8). Total length of replicate A F2 30-d-old carrier controls was significantly smaller than the 0.32 µg/L, 1.0 µg/L, 3.2 µg/L, and 10 µg/L treatments, and 0.10 µg/L was significantly smaller than 0.32 µg/L (Table 8). Total lengths in replicate B were similar with controls and significantly smaller than the carrier control, 0.10 µg/L, 1.0 µg/L, 3.2 µg/L, and 10 µg/L treatments, and 0.32 µg/L was significantly smaller than 1.0 µg/L (Table 8).

Table 2: Abiotic factors measured on lab water collected through-out the full life-cycle experiments. Values are written as means ± standard error.

Parameter	**Mean ± Std. Error**
Temperature (ºC)	26.0 ± 0.03
pH	7.83 ± 0.02
Conductivity (µS/cm)	121 ± 2.38
Dissolved Oxygen (ppm)	8.56 ± 0.06
Alkalinity (ppm as $CaCO_3$)	0.00
Hardness (ppm as $CaCO_3$)	0.00
Nitrite (ppm)	0.00
Nitrate (ppm)	0.00
Chlorine (ppm)	0.00

Table 3: Mortality of 7-8-d-old flagfish exposed to a 4-h pulse-exposure of endosulfan on day 47 of the full life-cycle experiment. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

Concentration (µg/L)	n	Mortality (%)
Control Water (0)	60	30.0[a]
Control Carrier (0)	60	26.7[a]
0.10	60	21.7[a]
0.32	60	28.3[a]
1.0	60	25.0[a]
3.2	60	21.7[a]
10	60	48.3[b]

Table 4: Length of 7-8-d-old flagfish on day zero of the full life-cycle experiment exposed to a 4-h pulse-exposure of endosulfan. The results of two replicates were pooled. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

Concentration (µg/L)	n	Total Length (mm) ± Std. Error
Control (0)	32	4.72 ± 0.08^{ab}
Carrier (0)	32	4.75 ± 0.05^{ab}
0.10	32	4.99 ± 0.08^{a}
0.32	32	4.86 ± 0.08^{ab}
1.0	32	4.62 ± 0.07^{b}
3.2	32	4.71 ± 0.08^{ab}
10	32	4.86 ± 0.10^{ab}

Table 5: Length of 56-57-d-old flagfish on day 49 of the full life-cycle experiment exposed to a 4-h pulse-exposure of endosulfan. The results of two replicates were pooled. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

Concentration (µg/L)	n	Total Length (mm) ± Std. Error
Control (0)	30	8.48 ± 0.23^{b}
Carrier (0)	30	8.46 ± 0.28^{b}
0.10	30	8.25 ± 0.22^{b}
0.32	30	7.83 ± 0.19^{ab}
1.0	30	7.33 ± 0.22^{a}
3.2	30	7.71 ± 0.25^{ab}
10	22	7.66 ± 0.28^{ab}

Table 6: Wet weight and total length of 135-136-d-old flagfish on day 128 of the full life-cycle experiment exposed to a 4-h pulse-exposure of endosulfan. The results of two replicates were pooled. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

Concentration (µg/L)	n	Wet Weight (g) ± Std. Error	Total Length (mm) ± Std. Error
Control (0)	30	1.18 ± 0.08^{ac}	39.6 ± 0.86^{ac}
Carrier (0)	29	0.88 ± 0.06^{b}	35.8 ± 0.99^{b}
0.10	34	0.90 ± 0.05^{b}	36.5 ± 0.84^{b}
0.32	26	0.91 ± 0.07^{b}	36.2 ± 0.92^{b}
1.0	27	1.11 ± 0.07^{abc}	39.0 ± 0.89^{abc}
3.2	31	0.92 ± 0.06^{b}	36.3 ± 0.79^{b}
10	18	1.35 ± 0.07^{ac}	40.5 ± 0.72^{ac}

Table 7: Wet weight and total length for 175-176-d-old flagfish (breeding harem fish) on day 168 of the full life-cycle experiment exposed to a 4-h pulse-exposure of endosulfan. The results of two replicates were pooled. Values are given as means ± standard error. There were no statistically significantly differences ($p \leq 0.10$).

Concentration (µg/L)	n	Wet Weight (g) ± Std. Error	Total Length (mm) ± Std. Error
Control (0)	12	1.83 ± 0.17	45.0 ± 1.15
Carrier (0)	12	1.88 ± 0.14	44.5 ± 1.21
0.10	12	1.95 ± 0.17	46.3 ± 1.39
0.32	12	2.21 ± 0.16	47.4 ± 1.03
1.0	12	2.04 ± 0.20	47.3 ± 1.47
3.2	12	1.88 ± 0.16	45.3 ± 1.22
10	12	2.21 ± 0.23	47.3 ± 1.40

Table 8: Wet weight and total length for 30-d-old post hatch larval flagfish obtained from parents given a 4-h pulse-exposure of endosulfan. The results could not be pooled. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$). Lettering pertains to each section individually.

	Concentration (µg/L)	n	Wet Weight (g) ± Std. Error	Total Length (mm) ± Std. Error
Replicate A	Control (0)	27	0.017 ± 0.001^{ab}	10.1 ± 0.199^{ab}
	Carrier (0)	25	0.013 ± 0.001^{a}	9.44 ± 0.224^{a}
	0.10	24	0.014 ± 0.001^{a}	9.96 ± 0.195^{a}
	0.32	29	0.021 ± 0.001^{b}	10.7 ± 0.228^{b}
	1.0	30	0.018 ± 0.001^{b}	10.4 ± 0.157^{b}
	3.2	29	0.018 ± 0.001^{b}	10.4 ± 0.196^{b}
	10	30	0.019 ± 0.001^{b}	10.6 ± 0.122^{b}
Replicate B	Control (0)	22	0.010 ± 0.001^{a}	9.18 ± 0.170^{a}
	Carrier (0)	28	0.019 ± 0.001^{d}	10.2 ± 0.186^{bc}
	0.10	30	0.016 ± 0.001^{bd}	10.3 ± 0.160^{bc}
	0.32	28	0.013 ± 0.001^{ac}	9.57 ± 0.227^{ac}
	1.0	25	0.018 ± 0.001^{bd}	10.6 ± 0.232^{b}
	3.2	30	0.019 ± 0.001^{bd}	10.2 ± 0.225^{bc}
	10	30	0.017 ± 0.001^{bd}	10.2 ± 0.180^{bc}

4.5.4 Condition Indices

There were no significant differences between replicates in condition factor of 135-136-d-old flagfish so the data were pooled, and re-analysis showed no significant differences between treatments; the condition factor ranged from 1.79-2.00 (Table 9).

The hepatosomatic index (HSI) had no significant differences between replicates so the data were pooled for interpretation. There was a significant decrease in the HSI for fish pulse-exposed to 0.1 µg/L when compared to the control water. However, none of the treatments were significantly different from the carrier control (Figure 9). The mean HSI was 2.03 ± 0.24.

Data were analyzed and there were no significant differences in the gonadosomatic index (GSI) between replicates so the data were pooled for interpretation. Fish pulse-exposed to 0.1 µg/L endosulfan had a significantly decreased GSI compared exposed to the water control, 0.32 µg/L, 3.2 µg/L and 10 µg/L (Figure 10). There were no significant differences between any of the treatments and the carrier control (Figure 10). The mean GSI was 2.39 ± 0.28. The overall NOEC and LOEC for this life-cycle study were thus 3.2 µg/L and 10 µg/L, respectively, due to increased mortality.

Table 9: Condition factor for 135-136-d-old flagfish on day 128 of the full life-cycle experiment exposed to a 4-h pulse-exposure of endosulfan. Values are given as means ± standard error. There were no statistically significantly differences ($p \leq 0.10$).

Concentration(µg/L)	n	Condition Factor ± Std. Error
Control (0)	30	1.87 ± 0.09
Carrier (0)	29	1.80 ± 0.05
0.10	34	1.78 ± 0.03
0.32	26	1.79 ± 0.08
1.0	27	1.83 ± 0.05
3.2	31	1.85 ± 0.07
10	18	2.00 ± 0.06

Figure 9: Hepatosomatic index of 135-136-d-old flagfish exposed to a 4-h pulse of endosulfan. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

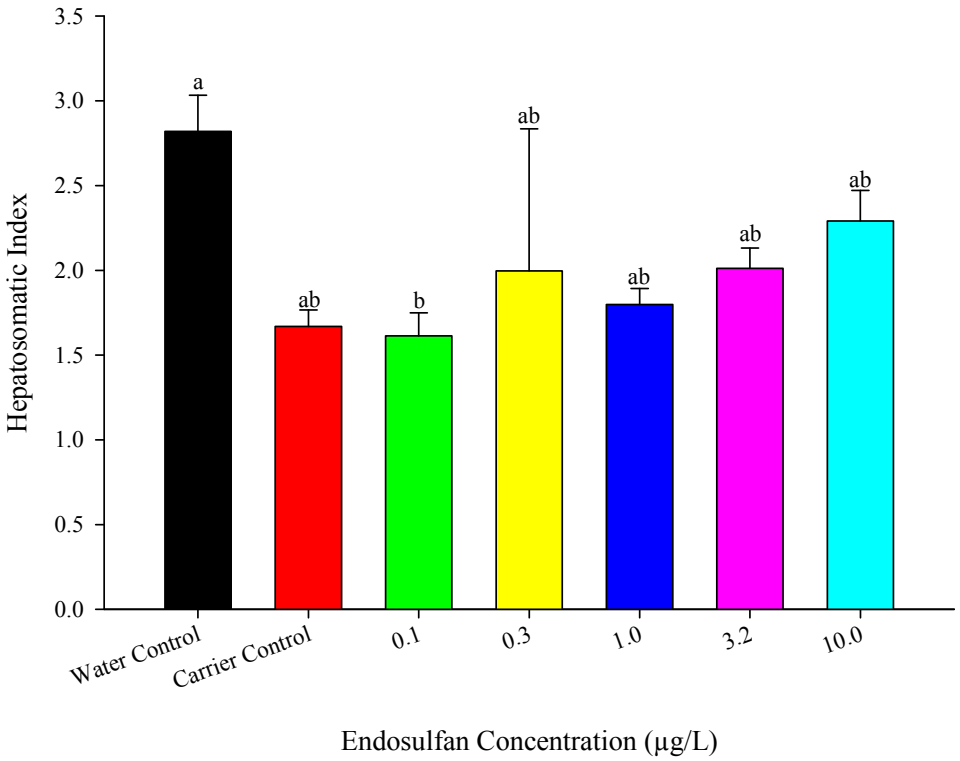
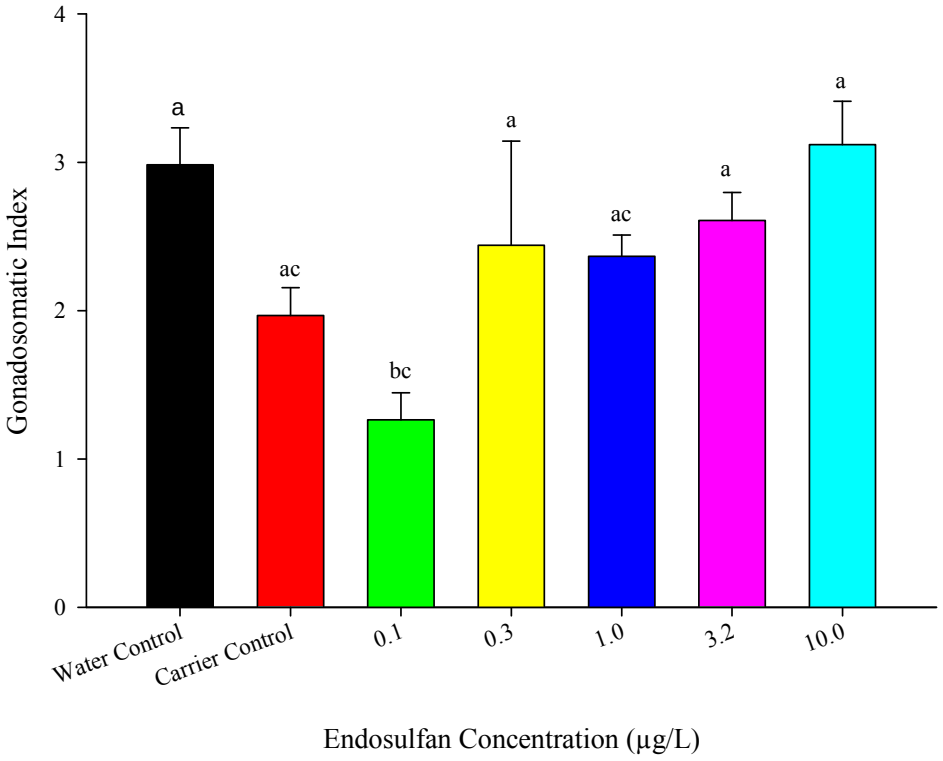


Figure 10: Gonadosomatic index of 135-136-d-old flagfish exposed to a 4-h pulse of endosulfan. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).



4.5.5 Reproductive Activity

The reproductive activity of flagfish pulse-exposed for 4-h to varying concentrations of endosulfan had significant differences so the data were analyzed separately. The time to first egg laying was not affected by the pulse-exposure and on average the flagfish laid their first egg on day 136 ± 1.4 (Table 10). There was little difference between controls and pulse-exposed fish in the number of days of eggs laid with an average of 19.7 ± 1.6 days of eggs laid, except for replicate A at 3.2 µg/L which only laid eggs for 9-d (Table 10). There was no significant difference between the controls and pulse-exposed flagfish in time to steady spawning with it taking on average 147 ± 1.2 days to reach steady spawning (Table 10).

Daily egg production for replicate A had significant differences within treatments, but of importance had a significant decrease in the amount of eggs produced at 3.2 µg/L or 10 µg/L compared to the carrier control (Table 10). Replicate B also had significant differences within treatments however there were no significant increases or decreases in any of the treatments compared to the control carrier (Table 10).

An interesting trend to note was that flagfish exposed to 0.10 µg/L in replicate A and flagfish exposed to 0.32 µg/L in replicate B had the highest number of days of eggs laid, the highest mean daily egg production, the highest total number of eggs produced, and the earliest time to reaching steady spawning compared to the controls (Table 10).

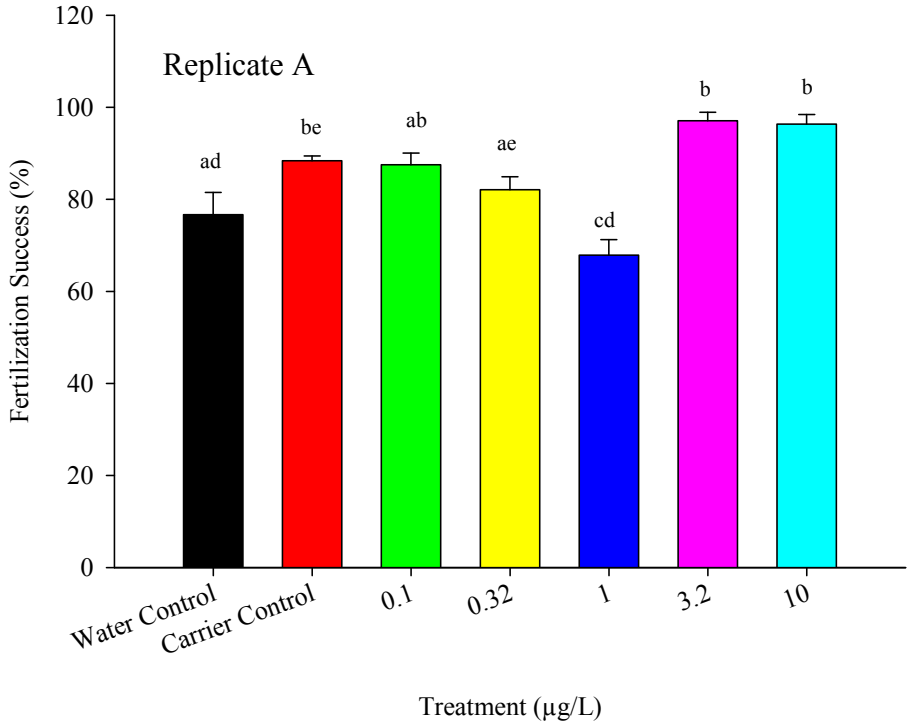
There were significant differences between replicates in fertilization success precluding the pooling of replicate data. Overall, there was quite a bit of variability between the treatments with regards to fertilization success. Replicate A fertilization success was significantly lower in the water control compared to the carrier control, 3.2

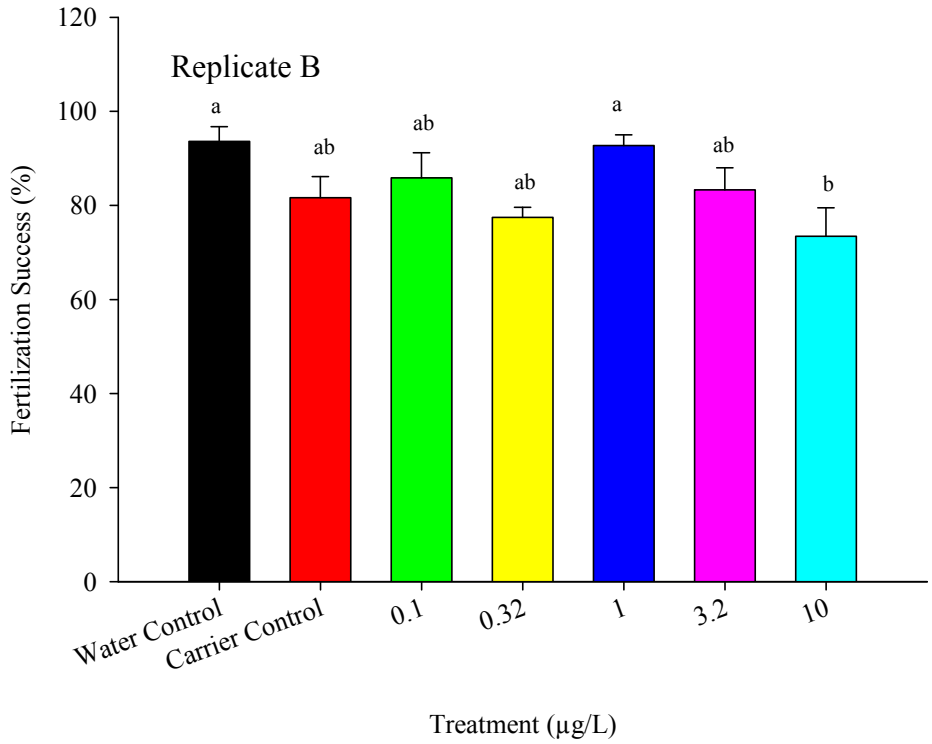
µg/L, and 10 µg/L treatment (Figure 11A). Replicate A also had a significantly lower fertilization success in the 1.0 µg/L treatment compared to the carrier control, 0.10 µg/L, 0.32 µg/L, 3.2 µg/L and 10 µg/L treatment (Figure 11A). Replicate B fertilization success was significantly higher in the water controls and 1.0 µg/L treatment compared to the 10 µg/L treatment (Figure 11B).

Table 10: Impacts of 4-h pulse-exposure to endosulfan on the reproductive capacity of flagfish.Within each replicate values not followed by the same letter are significantly different.

	Concentration (µg/L)	Time to first egg laying (d)	Number of days of eggs laid (d)	Daily egg production (eggs/d) (mean ± std. error)	Total Number of eggs produced (eggs)	Time to steady spawning (d)
Replicate A	Control (0)	135	22	90.0 ± 15.2^{adg}	2791	145
	Carrier (0)	133	28	90.3 ± 13.3^{ae}	2798	143
	0.10	134	30	125 ± 12.5^{ag}	3864	138
	0.32	133	18	26.3 ± 8.54^{bef}	815	151
	1.0	133	20	30.6 ± 6.34^{cdeh}	949	148
	3.2	151	9	3.06 ± 1.32^{bf}	95	not achieved
	10	145	16	7.61 ± 2.10^{bfh}	236	not achieved
Replicate B	Control (0)	134	17	12.8 ± 3.32^{ace}	398	not achieved
	Carrier (0)	135	13	26.6 ± 8.57^{ad}	826	157
	0.10	134	22	32.7 ± 7.63^{acf}	1014	146
	0.32	133	30	105 ± 11.4^{bd}	3263	139
	1.0	133	13	10.2 ± 3.33^{a}	316	146
	3.2	134	20	62.9 ± 14.9^{bdc}	1950	151
	10	134	18	69.4 ± 15.3^{bdf}	2151	149
	Average	136 ± 1.4	19.7 ± 1.6	-	-	147 ± 1.2

Figure 11: Fertilization success of eggs collected from breeding harem (2 males and 4 females) of flagfish exposed to 4-h pulse-exposure of endosulfan. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).





There was no significant difference in hatchability between replicates so the data were pooled. The hatchability of eggs collected from the pulse-exposed flagfish was > 96% in all of the treatments (Table 11). Hatchability in the 3.2 µg/L treatment was significantly higher than for eggs collected from 0.10 µg/L treated fish (Table 11).

There was a significant difference between replicates for time to hatch between replicates so the replicates were analyzed separately. The time to hatch ranged from 7-9 days between the two replicates. In replicate A, eggs collected from flagfish exposed to 3.2 µg/L had a significantly decreased time of hatch (6.83 ± 0.17) when compared to the eggs collected from flagfish exposed to 0.32 µg/L (7.60 ± 0.40) (Table 12). However, contradictory to this in replicate B flagfish eggs collected from fish exposed to 3.2 µg/L had a significantly increased time to hatch when compared to the carrier control, 0.10 µg/L, and 1.0 µg/L treated fish (Table 12).

Table 11: Hatchability of eggs collected from flagfish pulse-exposed for 4-h to endosulfan. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$).

Concentration (µg/L)	Hatchability (%) ± Std. Error
Control (0)	96.7 ± 0.6^{ab}
Carrier (0)	97.9 ± 0.5^{ab}
0.10	96.5 ± 1.1^{b}
0.32	98.4 ± 0.6^{ab}
1.0	97.8 ± 0.7^{ab}
3.2	99.5 ± 0.3^{a}
10	97.8 ± 1.0^{ab}

Table 12: Hatch length for eggs collected from flagfish pulse-exposed for 4-h to endosulfan. The results of replicates could not be pooled. Values are given as means ± standard error. Values without an alphabetical superscript in common are significantly different ($p \leq 0.10$). Lettering pertains to each section individually.

	Concentration (µg/L)	Hatch (d) ± Std. Error
Replicate A	Control (0)	7.00 ± 0.00^{ab}
	Carrier (0)	7.00 ± 0.00^{ab}
	0.10	7.00 ± 0.00^{ab}
	0.32	7.60 ± 0.40^{a}
	1.0	7.33 ± 0.21^{ab}
	3.2	6.83 ± 0.17^{b}
	10	7.00 ± 0.00^{ab}
Replicate B	Control (0)	7.50 ± 0.29^{ab}
	Carrier (0)	7.00 ± 0.00^{b}
	0.10	7.17 ± 0.17^{b}
	0.32	7.80 ± 0.20^{ab}
	1.0	7.00 ± 0.00^{b}
	3.2	8.00 ± 0.32^{a}
	10	7.60 ± 0.24^{ab}

5.0 Discussion

5.1 Toxicity of Endosulfan

Many other studies have been conducted to find acute toxicity values for exposure of endosulfan to various species of fish. These acute studies have produced numerous LC_{50} values; however these values have a wide range (Naqvi and Vaishanavi, 1993). One reason for such variation can be due to the use of different methodologies to obtain these LC_{50} values. Whenever both flow-through and static assays were conducted for the same species, the flow-through acute toxicity values were always lower (Sunderam *et al*., 1992). For example, eastern rainbow fish *(Melanotaenia duboulayi)* had a 96-h LC_{50} value of 0.5 µg/L for a flow-through test, and 11.4 µg/L for a static test (Sunderam *et al*., 1992). Keeping this in mind and the fact that LC_{50} values are typically completed on adult fish not larval fish here are some of the reported LC_{50} values for endosulfan with other species of fish.

The 96-h LC_{50} values for three freshwater catfishes; Bloch (*Mystus vittatus*), Hamilton (*Mystus cavasius*) and Bloch (*Heteropneustes fossilis*) using a continuous flow-through system were; 2.2 µg/L, 1.9 µg/L, and 1.1 µg/L respectively (Rao and Murty, 1982). Another freshwater fish *Macrognathus aculeatum* was exposed to endosulfan by a continuous flow-through system as well and was found to have a 96-h LC_{50} value of 3.5 ± 0.2 µg/L (Rao *et al*., 1981).

In a study conducted by Sunderam *et al*., (1992) various species of adult fish were exposed to a range of endosulfan concentrations under semistatic conditions and were monitored for mortality over 96-h. The semistatic testing was the same methodology employed for the 96-h LC_{50} experiment conducted in this research, thus the values

obtained by Sunderam *et al*., (1992) would be comparable to the flagfish results. The 96-h LC_{50} values found were; 0.6 µg/L for European carp (C*yprinus carpio*), 1.3 µg/L for bony bream (*Nematolosa erebi*), 1.2 µg/L for golden perch (*Macquaria ambigua*), 5.7 µg/L for silver perch (*Bidyanus bidyanus*), and 5.9 µg/L for eastern rainbow fish (*Melanotaenia duboulayi*) (Sunderam *et al*., 1992). In comparing the 4.35 µg/L 96-h LC_{50} value for larval flagfish (Figure 2) to these other species, larval flagfish can be classified as having an intermediary sensitivity to endosulfan.

A 168-h LC_{50} value from a 4-h pulse-exposure of larval flagfish to endosulfan was also determined to be 49.7 µg/L (Figure 5). Much of the LC_{50} literature available are reported at a standard 96-h time period using continuous exposure of adults. An extensive literature search did not find any other comparable data for a 4-h endosulfan pulse-exposure 168-h LC_{50} for flagfish or other species. However, 2-h pulse-exposure 96-h LC_{50} values for flagfish to another organochlorine insecticide, methoxychlor, were found to be 3.2 mg/L for 2-d-old fish, 13.5 mg/L for 4-d-old fish, and 38.6 mg/L for 8-9-old fish (Holdway and Dixon, 1985). Even though the pulse-exposure timing and duration of the experiment were different, the fact that methoxychlor produced an LC_{50} in the ppm versus endosulfan which produced an LC_{50} value in the ppb makes it clear that larval flagfish are more sensitive to endosulfan than methoxychlor, because it takes a much smaller amount of endosulfan than methoxychlor to be toxic. This may be due to the amount of chlorine that is present in endosulfan as opposed to methoxychlor.

Another study that had a similar methodology of pulse-exposure to larval fish examined the effects of cadmium and zinc on hatchability, larval development, and survival of Australian crimson spotted rainbow fish (*Melantoaenia fluviatilis*) (Williams

and Holdway, 2000). The experiment generated 2-h pulse-exposure LC_{50} values for 9-10-d-old fish for cadmium of 0.56 mg/L and for zinc 1.57 mg/L (Williams and Holdway, 2000). They also reported a continuous exposure LC_{50} value of 0.01 mg/L for cadmium and 0.27 mg/L for zinc (Williams and Holdway, 2000). These findings help to demonstrate that pulse-exposures require a higher amount of toxicant to reach the LC_{50} than a continuous exposure, due to the shorter exposure time available for absorption. This is in agreement with the LC_{50} value of 4.35 µg/L for a 96-h continuous exposure and 49.7 µg/L for a 4-h pulse-exposure observed in this study (Figure 2, 5).

From the LC_{50} data obtained, a 96-h continuous exposure NOEC value of 3.2 µg/L and a LOEC value of 10 µg/L were found (Figure 3). The life-cycle 4-h pulse-exposure NOEC and LOEC were 3.2 and 10 µg/L endosulfan, respectively, due to significant mortality in the highest test concentration of 10 µg/L. A 4-h pulse-exposure of endosulfan to adult crimson-spotted rainbow fish found the NOEC value to be < 1.0 µg/L and the LOEC value to be 1.0 µg/L (Holdway *et al*., 2008), somewhat lower than the 3.2 µg/L NOEC reported here. With this comparison in mind, although NOECs and LOECs are useful measures of toxicity, they are very dependent on the design of the experiment rather than the toxicity of the chemical only (Chapman *et al*., 1998). Depending on the endpoints and concentrations chosen to be tested, the LOEC and NOEC values can vary greatly (Chapman *et al*., 1998). As was seen with the 4-h pulse-exposure experiment, the range of concentrations chosen was unable to produce a LOEC. If a wider range of concentrations had been chosen when designing the experiment, a more accurate LOEC could have been determined.

A high percentage mortality in the early stages of the life-cycle was observed and this was likely due to malnutrition. The lack of feeding coupled with the sub-lethal effects of a 4-h pulse-exposure of endosulfan to larval flagfish led to a significantly higher percentage of mortality in the 10 μg/L treatment compared to all other treatments (Table 3).

5.2 Sub-lethal Behavioural Effects of Endosulfan

Observed sub-lethal effects from endosulfan exposure of larval flagfish were hyperactivity, convulsions, immobility, and axis malformation (Figure 7-8). Juvenile catfish (*Clarias batrachus*) exposed to a concentration range of 0.005 to 0.04 mg/L of endosulfan in a 96-h static bioassay were observed to undergo frequent jumping, erratic movement, convulsions, increased opercular rate, and loss of equilibrium followed by death (Gopal *et al*., 1981). The Asian swamp eel (*Monopterus albus*, Zuiew) also exhibited these behaviours when exposed to a concentration range of 0.01 to 10 μg/L of endosulfan for 96-h (Siang *et al*., 2007).

Severe abnormal behaviour in flagfish was mostly observed at endosulfan concentrations > 3.2 μg/L, but some hyperactivity and convulsions were still noted at that concentration. This value is in close agreement with the NOEC of 0.00316 mg/L that was reported for juvenile catfish exposed to endosulfan (Gopal *et al*., 1981).

Abnormal sub-lethal behaviour previously described for fish exposed to endosulfan can be explained by its mode of action. As discussed earlier, endosulfan acts directly on the central nervous system and binds to the picrotoxin site in the GABA complex (Coats, 1990; Harris *et al*., 2000). When endosulfan binds to the picrotoxin site

it impairs the normal function of the GABA channel (chloride flux is inhibited) and causes hyperexcitation (Coats, 1990; Gant *et al*., 1987; Harris *et al*., 2000). This imbalance results in the hyperexcitability, convulsions, and in some cases mortality that occurred in larval flagfish following endosulfan exposure.

It is also important to note that recovery from endosulfan exposure was observed in some of the sub-lethal exposures (Figure 8). An explanation for the recovery of flagfish could be due to the short duration of the pulse to endosulfan, and the transfer of larval fish to freshwater immediately after exposure allowing for a faster elimination of endosulfan and its metabolites (Toledo and Jonsson, 1992).

Sub-lethal effects caused by endosulfan exposure in laboratory experiments are of great concern for the survival of fish in the wild, as they could lead to increased predation and an inability to acquire food (Ballesteros *et al*., 2009; Hiran and Arends, 2003). Since the life-cycle study monitored reproductive effects, concentrations needed to be selected that would not kill larval flagfish, and thus prevent them from reaching maturity. Preliminary sub-lethal studies indicated that a concentration of 10 µg/L should be selected as the highest test concentration used in the full life-cycle experiment.

5.3 Effect of Endosulfan on Condition Indices

Other sub-lethal effects studied in the full life-cycle study were the condition factor, GSI, HSI, and growth. The condition factor is a measure commonly used to assess the health of the treated fish in comparison to the controls; it is based on a ratio between body length and weight (Schlenk *et al*., 2008; van der Oost *et al*., 2003). No significant difference was seen in any of the treatments and thus there were no observed

toxic effects on the overall health of the flagfish from exposure to sub-lethal levels of endosulfan.

The HSI had a significant difference between 0.1 µg/L and the water control; however, there was no significant differences between any of the treatments and the carrier control (Figure 9). HSI is a very common measurement often used in toxicology assessments to evaluate the effect of toxicants on the liver; it is a ratio of the liver weight to body weight (Schlenk *et al*., 2008). Typically, liver enlargement can be indicative of contaminant exposure and may be due to hypertrophy or hyperplasia (van der Oost *et al*., 2003). Since the liver is the main site of detoxification for endosulfan in fish, a consistent difference in HSI would provide evidence of an effect on the liver (Nowak and Ahmad, 1989; Rao and Murty, 1982). Since HSI can be affected by other things such as nutrition and disease, it is not always the most reliable indicator but can be used as a pre-screening tool to then further investigate the effects with a more detailed approach (van der Oost *et al*., 2003). Much of the variability seen in the levels of HSI was likely due to individual fish variation within treatments.

The GSI showed some significant differences within the treatments but had no common trend or pattern between the treatments (Figure 10). GSI is a ratio of gonad weight to body weight, it represents the reproductive status of fish and it is a common endpoint used in toxicology assessment (Brewer *et al*., 2008; Rinchard and Kestemont, 1996; Schlenk *et al*., 2008). GSI was examined to see if endosulfan exposure would have a direct effect on the gonads. There were no significant differences in GSI from carrier controls while minor variable differences were seen between 0.1 µg/L and water control, 0.32 µg/L, 3.2 µg/L, and 10 µg/L. Flagfish are multi-spawners, meaning they lay

multiple clutches of eggs over time. Studies looking at the use of GSI for multi-spawners have observed that it is of limited use and can be an unreliable indicator; fish can be in different stages depending on whether or not they have just spawned (Brewer *et al*., 2008; Rinchard and Kestemont, 1996). Thus, GSI is not the most accurate or reliable technique to use for multi-spawning fish and the variation observed in flagfish GSI levels appears to support this.

Growth is an easy and cost effective measurement that can be used to study the effects of toxicants on fish (Schlenk *et al*., 2008). The slight differences seen in the initial growth measurements were due to randomization. Fish were pooled and then randomly allocated into crystallization dishes which removed any bias and allowed for true randomization to be the cause of an uneven distribution of fish length. Growth can be affected by many different things including, but not limited to, water temperature, food intake, and density (Schlenk *et al*., 2008). The slightly larger size of 10 µg/L treated fish at 135-136-d-old was likely a factor of density, since fish growth is very density dependent (Schlenk *et al*., 2008).

Overall no significant growth effects were seen from a 4-h pulse-exposure of endosulfan to larval flagfish at concentrations up to and including 10 µg/L. This was also demonstrated in an experiment in which Japanese medaka (*Oryzias latipes*) were exposed to 0.01, 0.1, and 1.0 µg/L of endosulfan for 24-h either just after fertilization, or shortly after hatching (Gormley and Teather, 2003). They found that larvae hatched from eggs exposed to endosulfan had reduced growth but fry exposed to endosulfan did not show any differences in length (Gormley and Teather, 2003).

5.4 Reproductive Effects of Endosulfan

The reproductive parameters assessed in this life-cycle experiment were not very sensitive to a 4-h pulse-exposure of endosulfan up to 10 µg/L (Table 10). While flagfish daily egg production was significantly decreased in the 3.2 and 10 µg/L treatment in replicate A compared to the carrier control, there were no significant differences seen in those concentrations for replicate B. The amount of variability seen between the two replicates with regards to egg production makes it hard to conclude any definite effects, and thus more experiments would need to be run in order to conclusively state whether or not there are reproduction impairments due to exposure of endosulfan at concentrations of 3.2 µg/L and 10 µg/L.

In a similar study performed by Gormley and Teather, (2003) exposed Japanese medaka (*Oryzias latipes*) to environmentally realistic concentrations of 0.01, 0.1, and 1.0 µg/L for 24-h either just after fertilization, or shortly after hatching. They studied the growth, survivorship, mobility, foraging ability and reproduction effects of this exposure (Gormley and Teather, 2003). Flagfish saw an increase in egg production in 0.1 µg/L for replicate A and in 0.32 µg/L for replicate B, while the number of medaka eggs produced in the 0.01 and 1.0 µg/L treatment were significantly higher than in the controls, while there was no difference in the 0.1 µg/L treatment (Gormley and Teather, 2003). Thus, the effects of endosulfan on egg production were not dose-dependent in these studies and different concentrations and different species may elicit different effects in egg production.

Flagfish treated with 0.10 µg/L in replicate A and 0.32 µg/L in replicate B of endosulfan had the greatest number of days with eggs laid, had the highest egg

production per day, had the highest total number of eggs produced, and were the earliest fish to reach steady spawning (Table 10). These effects could be due to a low- dose stimulation, however due to the amount of variability between replicates another experiment would have to be run in order to make any definite conclusions (Calabrese, 1999; Calabrese, 2008; Weltje *et al*., 2005).

The fertilization success of pulse-exposed flagfish saw a significant reduction in the 10 µg/L treatment compared to the control water and 1 µg/L treatment (Figure 11A, B). Possible explanations for this decrease in fertilization could be due to interruptions in mating behaviour, or decreased sperm motility. Flagfish exhibit extensive mating behaviour as described previously, and since flagfish are oviparous, disturbances in the actual mating act could lead to a reduction in the number of fertilized eggs. Since this observation was not consistently seen in all endosulfan treatments nor was it different from the carrier controls, further investigation into changes in reproductive behaviour seen or sperm motility would be needed.

The eggs collected from flagfish exposed to a 4-h pulse of endosulfan showed no significant differences from the controls in hatchability (Table 11). However, there were some significant differences observed in the time to hatch (Table 12). Overall, the time to hatch was between 7-9 days for the two replicates, with 3.2 µg/L showing both an increase and decrease (Table 12). Eggs collected from medaka that were exposed to 0.01, 0.1, and 1.0 µg/L of endosulfan as fry, had no significant differences in time to hatch (Gormley and Teather, 2003). In other experiments, flagfish eggs have been reported as hatching anywhere in the range of 4-7 days, however many factors can

influence the time to hatch such as temperature, and water parameters (Foster *et al*., 1969; Smith, 1973).

A recent study by Sarma *et al*., (2009) exposed the spotted murrel (*Channa punctatus*) to a sub-lethal level of 8.1 µg/L of endosulfan and studied how different diets could alter the effects of endosulfan. It was found that high crude protein and vitamin C diets helped to improve growth and metabolism and reduced the endosulfan bioaccumulation (Sarma *et al*., 2009). Although the life-cycle experiment in this study involved a short pulse-exposure and did not monitor the actual bioaccumulation of endosulfan, dietary influences could still be a possible explanation for the absence of observed growth or reproductive effects due to sub-lethal pulse-exposures of up to 10 µg/L of endosulfan to larval flagfish. The flagfish were fed a constant diet of flake food (high in crude protein), frozen brine shrimp, and freshly hatched brine shrimp on a frequent basis in large amounts in order to allow for faster growth (highly fed fish reach sexual maturity faster) and to keep the reproductive level high. This frequently available excess of food might have counteracted and reduced the overall effects of endosulfan on growth and reproduction by allowing for better detoxification of endosulfan (Sarma *et al*., 2009).

5.5 Limitations and Future Research

If time had permitted, a better method to measure the effects of endosulfan on gonads and liver might have utilized a histological analysis of the tissues collected (liver and gonads). Also, measuring the growth and reproduction of the offspring of pulse-exposed fish for a longer period of time may have provided more information about the

transfer effects of endosulfan exposure, if any, from parental generations to larval generations.

Many continuous exposure studies have been conducted on pesticides but in the environment, much exposure to pesticides is through agricultural run-off that often occurs in pulses. More research on pulse-exposure effects of pesticides to non-target organisms may help to better predict safe levels for water in order to avoid detrimental effects to non-target organisms. As well, more data needs to be generated on the standard measure of toxicity to larval and juvenile fish by determining and reporting both continuous exposure and pulse-exposure LC_{50}s.

Some questions left unanswered by this research are at what concentration would detrimental reproductive effects from endosulfan pulse-exposure manifest themselves? Also, to what extent does the duration of the pulse-exposure time period play on endosulfan toxicity?

6.0 Conclusion

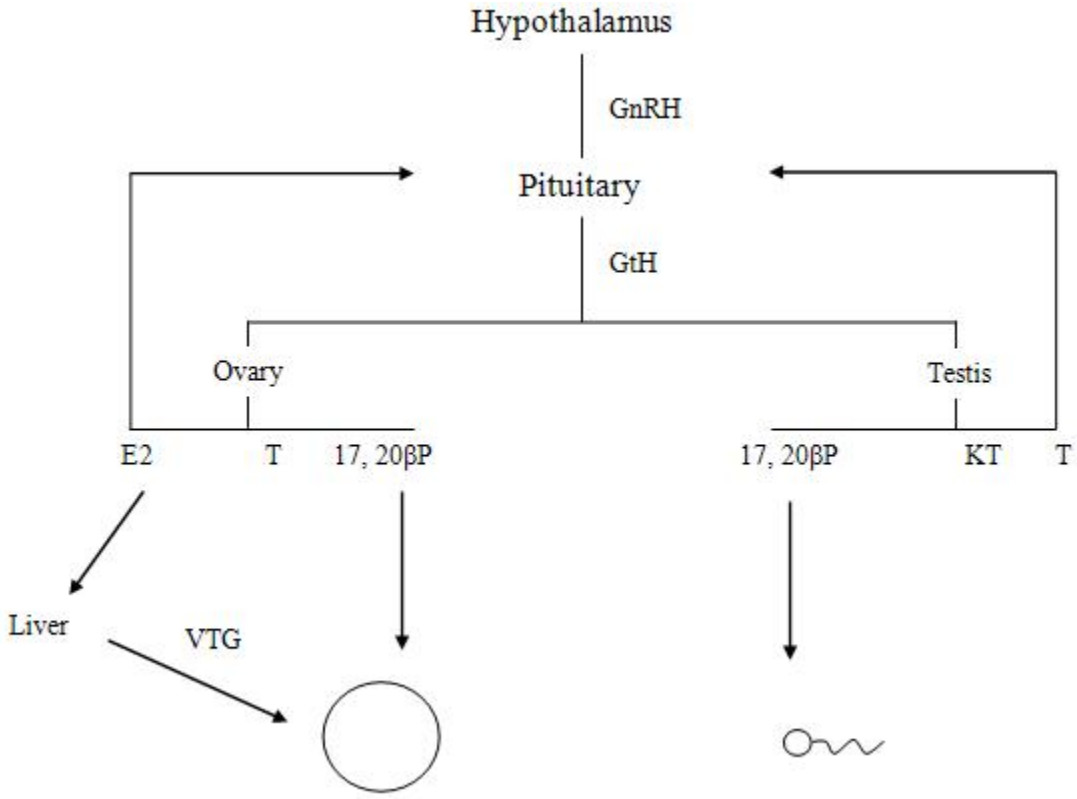
Assessing the effects of toxicants on organisms in the environment has been a major area of focus for many years, specifically the use of man-made pesticides and their effects on non-target organisms. The potential effects of these toxicants in an acute and chronic manner are of high importance and have fueled much research over the years.

This research studied the effects of continuous and pulse-exposure of endosulfan on larval flagfish. A major finding of this research was the 96 hour continuous exposure LC_{50} in larval flagfish of 4.35 µg/L and a 4-h pulse-exposure LC_{50} value for larval flagfish of 49.7 µg/L. Various observations on the sub-lethal effects included hyperactivity, convulsions, and some axis malformation.

Finally, the effects of a 4-h endosulfan pulse-exposure up to 10 µg/L on 7-8 d-old larval flagfish showed no significant growth, or reproductive effects when investigated over a full life-cycle and significant mortality only at 10 µg/L. Thus, a 4-h pulse-exposure of endosulfan in the environment at a concentration of $\leq$ 3.2 µg/L likely has no significant effect on the freshwater species *Jordanella floridae*.

Appendix 1

The basic reproductive physiology system in fish. (GnRH = gonadotrophin releasing hormone; GtH = gonadotrophin; E2 = estradiol; T = testosterone; 17,20βP = 17, 20 β-dihydroxy-4-pregnen-3-one; KT = 11-ketotestosterone) (Adapted from Kime *et al*., 1995).



Appendix 2

Nominal and measured concentrations of endosulfan for full life-cycle study.

Set	Nominal	Actual	Nominal (%)
A	0	0.00	Not Detected
A	0	0.00	Not Detected
A	0.10	0.16	160
A	0.32	0.42	131
A	1.0	2.19	219
A	3.2	2.91	91
A	10	12.3	123
B	0	0.00	Not Detected
B	0	0.00	Not Detected
B	0.10	0.23	230
B	0.32	0.50	156
B	1.0	1.58	158
B	3.2	3.69	115
B	10	9.30	93

Appendix 3

Timeline of activities and assessments during a full life-cycle study of a pulse-exposure of endosulfan to Flagfish.

Time	Activity	Assessment
N/A Observations	Collection of eggs (F2) to be used for pulse-exposure	Daily
N/A	Hatching of larval flagfish (F2)	# of eggs
Day 0	4-h pulse-exposure of 30 larval flagfish (F2) to each concentration of endosulfan	Mortality, Growth Photo
~Day 48	Juveniles switched from crystallization dishes to 10L tanks	Mortality, Growth Photo
~Day 87	Juveniles (F2) moved to 70 L tank, breeding substrate added to each tank	Mortality
~Day 128	Thinned, selection of fish (F2) for breeding harem; 2 males and 4 females per tank	Mortality, Growth, GSI, HSI
~Day 133-163	Daily Collection of eggs (F3)	Time to first spawn Time to steady spawning # of eggs, Hatchability Fertilization
~Day 168	Euthanize 4-month-old flagfish (F2)	Growth

F0= Parental Fish, F1= First Generation, F2= Second Generation

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